



# UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE  
United States Patent and Trademark Office  
Address: COMMISSIONER FOR PATENTS  
P.O. Box 1450  
Alexandria, Virginia 22313-1450  
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
10/032,717	10/23/2001	Andre R. Abad	35718/237005 (5718-118)	5409
826	7590	03/06/2007		
ALSTON & BIRD LLP BANK OF AMERICA PLAZA 101 SOUTH TRYON STREET, SUITE 4000 CHARLOTTE, NC 28280-4000			EXAMINER KUBELIK, ANNE R	
			ART UNIT	PAPER NUMBER
			1638	
SHORTENED STATUTORY PERIOD OF RESPONSE		MAIL DATE	DELIVERY MODE	
2 MONTHS		03/06/2007	PAPER	

**Please find below and/or attached an Office communication concerning this application or proceeding.**

If NO period for reply is specified above, the maximum statutory period will apply and will expire 6 MONTHS from the mailing date of this communication.



UNITED STATES PATENT AND TRADEMARK OFFICE

---

Commissioner for Patents  
United States Patent and Trademark Office  
P.O. Box 1450  
Alexandria, VA 22313-1450  
[www.uspto.gov](http://www.uspto.gov)

**BEFORE THE BOARD OF PATENT APPEALS  
AND INTERFERENCES**

Application Number: 10/032,717  
Filing Date: October 23, 2001  
Appellant(s): ABAD ET AL.

**MAILED**  
**MAR 06 2007**  
**GROUP 1600**

---

W. Murray Spruill  
For Appellant

**EXAMINER'S ANSWER**

This is in response to the appeal brief filed 17 November 2006 appealing from the Office  
action mailed 18 May 2006

**(1) Real Party in Interest**

A statement identifying by name the real party in interest is contained in the brief.

**(2) Related Appeals and Interferences**

The following are the related appeals, interferences, and judicial proceedings known to the examiner that may be related to, directly affect or be directly affected by or have a bearing on the Board's decision in the pending appeal:

Application Serial Number 10/414,637, which is a divisional of the instant application.

**(3) Status of Claims**

The statement of the status of claims contained in the brief is correct.

**(4) Status of Amendments After Final**

The appellant's statement of the status of amendments after final rejection contained in the brief is correct.

**(5) Summary of Claimed Subject Matter**

The summary of claimed subject matter contained in the brief is correct.

**(6) Grounds of Rejection to be Reviewed on Appeal**

The appellant's statement of the grounds of rejection to be reviewed on appeal is correct.

Art Unit: 1638

**(7) Claims Appendix**

The copy of the appealed claims contained in the Appendix to the brief is correct.

**(8) Evidence Relied Upon**

Evidence Appendix A: sequence comparison between SEQ ID NOs:2 and 4.

Evidence Appendix B: Tounsi et al, 2003, J. Appl. Microbiol. 95:23-28.

Evidence Appendix C: Minshull et al

Evidence relied upon and provided by Appellant:

Lazar et al, 1988, Mol. Cell. Biol. 8:1247-1252: see Appellant's Appendix B.

Hill et al, 1998, Biochem. Biophys. Res. Comm. 244:573-577: see Appellant's Appendix C.

de Maagd et al, 1999, Appl. Environ. Microbiol. 65:4369-4374: see Appellant's Appendix D.

Angsuthanasombat et al, 2001, J. Biochem. Mol. Biol. 34:402-407: see Appellant's Appendix E.

Li et al: see Appellant's Appendix G.

**(9) Grounds of Rejection**

The following ground(s) of rejection are applicable to the appealed claims:

- a. Claims 1-3, 9-12, 17-19, 38, 43, 46, 49, 52 and 55-64 are rejected under 35

Art Unit: 1638

U.S.C. 112, first paragraph, because the specification, while being enabling for nucleic acids encoding SEQ ID NO:2 and 10, expression cassettes comprising the nucleic acids, plants and seeds comprising a construct comprising the nucleic acid, and a method of using it to impact a plant pest, does not reasonably provide enablement for any nucleic acid that has 90% identity to SEQ ID NO:1, expression cassettes comprising the nucleic acid, plants and seeds comprising a construct comprising the nucleic acid and a method of using it to impact a plant pest. The specification does not enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention commensurate in scope with these claims.

The claims are broadly drawn to a nucleic acid that has 90%, 93%, 94% or 95% identity to SEQ ID NO:1 and that encodes a Coleopteran pesticidal protein, expression cassettes comprising the nucleic acids, plants and seeds comprising constructs comprising the nucleic acids, and a method of using the constructs to impact a plant pest.

The instant specification, however, only provides guidance for methods of assaying the activity of *B. thuringiensis* strain 1218 and lysate against Western corn rootworm and Southern corn rootworm (examples 1 and 2); isolation of crystal protein from the strain and assaying of it for pesticidal activity against western corn rootworm (example 3); identification of two coding regions, *Cry1218-1* and *Cry1218-2* (SEQ ID NO:1 and 3, respectively, with SEQ ID NOs:27 and 28 as the genomic clones), isolated by unknown methods, as encoding proteins, SEQ ID NOs:2 and 4, respectively, that have an unspecified homology to Cry8Ba1 (example 4); production in *E. coli* of the truncated proteins, SEQ ID NOs:15 and 17, encoded by SEQ ID NOs:16 and 18, respectively, that are active against southern corn rootworm (example 4); and production of

Art Unit: 1638

maize-preferred coding sequences of a different truncated version of Cry1218-1 - the nucleic acid is SEQ ID NO:9, which encodes SEQ ID NO:10 (example 5). The specification also teaches making three mutant versions of truncated Cry1218-1 (SEQ ID NO:16), one of which has a truncated N-terminus (comprising amino acids 43-663 of SEQ ID NO:16), and the others in which the 4 amino acid sequence NGSR or LKMS has been inserted after amino acid 164 or replacing amino acids 164-166 - all of these mutant proteins are more effective against southern and western corn rootworm than Cry1218-1 (examples 6-7). The specification also teaches transformation of maize with SEQ ID NO:9 (examples 8 and 9).

The instant specification fails to provide guidance for how to make nucleic acids that have 90%, 93%, 94% or 95% identity to the full-length of SEQ ID NO:1 and that encode a Coleopteran pesticidal protein. The instant specification thus also fails to provide guidance for how to make expression cassettes comprising the nucleic acids, plants and seeds comprising a construct comprising the nucleic acids and a method of using it to impact a plant pest.

The instant specification fails to provide guidance for the full scope of which amino acids of SEQ ID NO:2 can be altered and to which other amino acids, and which amino acids must not be changed, to maintain Coleopteran pesticidal activity of the encoded protein. The specification also fails to provide guidance for the full scope of which amino acids can be deleted and which regions of the protein can tolerate insertions and still produce a functional enzyme.

Because nucleic acids that have 90% identity to the 3621 nucleotide long SEQ ID NO:1 would have up to 362 nucleotide substitutions, they could encode proteins with up to 362 amino acid substitutions; these proteins would have 70% identity to the 1206 amino acid long SEQ ID NO:2. The specification provides no guidance for which 362 amino acids to substitute and still

Art Unit: 1638

maintain Coleopteran pesticidal activity. Thus, undue trial and error experimentation would be required to make the claimed nucleic acids.

Similarly, nucleic acids that have 93% identity to SEQ ID NO:1 would have up to 253 nucleotide substitutions, nucleic acids that have 94% identity to SEQ ID NO:1 would have up to 217 nucleotide substitutions and nucleic acids that have 90% identity to SEQ ID NO:1 would have up to 181 nucleotide substitutions, producing proteins with 79%, 82% and 85% identity, respectively, to the 1206 amino acid long SEQ ID NO:2.

The specification on pg 28, lines 5-16, suggests making these nucleic acids by making conservative substitutions in the encoded protein. However, making “conservative” substitutions (*e.g.*, substituting one polar amino acid for another, or one acidic one for another) in proteins does not produce predictable results. Lazar et al (1988, Mol. Cell. Biol. 8:1247-1252) showed that the “conservative” substitution of glutamic acid for aspartic acid at position 47 reduced biological function of transforming growth factor alpha while “nonconservative” substitutions with alanine or asparagine had no effect (abstract). Similarly, Hill et al (1998, Biochem. Biophys. Res. Comm. 244:573-577) teach that when three histidines that are maintained in ADP-glucose pyrophosphorylase across several species are substituted with the “nonconservative” amino acid glutamine, there is little effect on enzyme activity, while the substitution of one of those histidines with the “conservative” amino acid arginine drastically reduced enzyme activity (see Table 1). The nucleic acids encoding all these mutated proteins, however, would have much greater than 90% identity to the nucleic acids encoding the original protein.

Making amino acid substitutions in *cry* proteins is also unpredictable. Each *cry* protein only has activity against one or few insect species (de Maagd et al, 1999, Appl. Environ.

Art Unit: 1638

Microbiol. 65:4369-4374, see pg 4369, column 1, paragraph 1), and even conservative substitutions in nonconserved regions can have unexpected effects on protein function (Figs 2 and 3). Even a single amino acid substitution in a *cry* protein may alter its insecticidal specificity, and toxicity must be determined empirically (Tounsi et al, 2003, J. Appl. Microbiol. 95:23-28; see pg 27, column 2, paragraph 2). For example, a conservative substitution of a lysine for an arginine in a *cry11A* protein eliminated toxicity to *Aedes aegyptii* but not other pests to which the wild-type protein has toxicity (Angsuthanasombat et al, 2001, J. Biochem. Mol. Biol. 34:402-407, paragraph spanning the columns on pg 405).

The specification, on pg 65, lines 12-14, indicates that the instant SEQ ID NO:1 has homology to GenBank U04365, which is identical to SEQ ID NO:3 of Michaels et al (1996, US Patent 5,554,534). This nucleotide sequence has 85.1% identity to SEQ ID NO:1. The specification, on pg 29, suggests using GenBank U04365 (SEQ ID NO:3 of Michaels et al ) as the other nucleic acid in shuffling to create the claimed nucleic acids; however, that nucleic acid encodes a protein with 79.8% identity to the instant SEQ ID NO:2 (see sequence search report). Thus, this sequence cannot be used as guidance for nucleic acids with 90% identity to SEQ ID NO:1 and that encode proteins with 70% identity to SEQ ID NO:2, as encompassed by the full scope of the claims.

Furthermore, SEQ ID NO:3 of Michaels et al is pesticidal for *Cyclocephala borealis* (northern masked chafer) and *Popillia japonica* (Japanese beetle)(column 14, line 46, to column 15, line 2), whereas the instant SEQ ID NO:1 is pesticidal to *Diabrotica longicornis howardi* (southern corn rootworm). Given the teachings of Tounsi et al, de Maagd et al, and Angsuthanasombat et al, one of skill in the art could not even predict for which pests the protein



Art Unit: 1638

encoded by nucleic acids that have 90% identity to SEQ ID NO:1 would be toxic, and the specification does not teach which amino acid substitutions must can be made to retain southern corn rootworm pesticidal activity.

Given the claim breath, unpredictability, and lack of guidance as discussed above, undue experimentation would have been required by one skilled in the art to develop and evaluate nucleic acids with 90% identity to SEQ ID NO:1. a trail and error approach to making the claimed nucleic acids may require one to make all possible single amino acid substitutions in SEQ ID NO:1. Making all possible single amino acid substitutions in an 3621 nucleotide long nucleic acid like that of SEQ ID NO:1 would require making and analyzing  $19^{3621}$  nucleic acids (or  $2.3 \times 10^{4630}$  nucleic acids; for comparison, the estimate of the total number of atoms in the universe is  $10^{81}$ ). These nucleic acids would have about 99.99% identity to SEQ ID NO:1. Because nucleic acids that have 90% identity to SEQ ID NO:1 would have up to 362 nucleotide substitutions, many more than  $19^{3621}$  nucleic acids would need to be made and analyzed without further guidance.

Guo et al (2004, Proc. Natl. Acad. Sci. USA 101: 9205-9210) teach that while proteins are fairly tolerant to mutations resulting in single amino acid changes, increasing the number of substitutions additively increases the probability that the protein will be inactivated (pg 9209, right column, paragraph 2). Thus, making and analyzing proteins with up to 362 amino acid substitutions that also have pesticidal activity would require undue experimentation.

As the specification does not describe the transformation of any plant with nucleic acids with 90% identity to SEQ ID NO:1 and encoding Coleopteran pesticidal proteins within the full scope of the claims, undue trial and error experimentation would be required to screen through

Art Unit: 1638

the myriad of nucleic acids encompassed by the claims and plants transformed therewith, to identify those that could control plant pests, if such plants are even obtainable.

Given the claim breath, unpredictability in the art, undue experimentation, and lack of guidance in the specification as discussed above, the instant invention is not enabled throughout the full scope of the claims.

b. Claims 1-3, 9-12, 17-19, 38, 43, 46, 49, 52 and 55-64 are rejected under 35 U.S.C. 112, first paragraph, as containing subject matter that was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention.

A full review of the specification indicates that nucleic acids that have 90%, 93%, 94% or 95% identity to SEQ ID NO:1 and that encode Coleopteran-pesticidal proteins are essential to the operation of the claimed invention.

The level of skill and knowledge in the art at the time of filing was such that while large number of Coleopteran pesticidal proteins were known, nucleic acids that have 90%, 93%, 94% or 95% identity to SEQ ID NO:1 and that encode such Coleopteran-pesticides were not.

The claim is directed to a genus of nucleic acids that have 90%, 93%, 94% or 95% identity to SEQ ID NO:1. Nucleic acids that have 90%, 93%, 94% or 95% identity to SEQ ID NO:1 would have up to 362 nucleotide substitutions, they could encode proteins with up to 362, 253, 217 or 181 amino acid substitutions relative to the to the 1206 amino acid long SEQ ID NO:2.

The structural features that distinguish Coleopteran-pesticide-encoding nucleic acids that have 90%, 93%, 94% or 95% identity to SEQ ID NO:1 from other that have 90%, 93%, 94% or

Art Unit: 1638

95% identity to SEQ ID NO:1 are not described in the specification. The specification recites no structure required for Coleopteran-pesticide activity. The necessary and sufficient structural elements of a protein with Coleopteran-pesticidal activity are not described.

The only species described in the specification is SEQ ID NO:1, which encodes SEQ ID NO:2, a truncated version of SEQ ID NO:1, and a few nucleic acids with a few nucleotide substitutions relative to SEQ ID NO:1.

One of skill in the art would not recognize that Applicant was in possession of the necessary common attributes or features of the genus in view of the disclosed species. Since the disclosure fails to describe the common attributes that identify members of the genus, and because the genus is highly variant, SEQ ID NO:1 alone is insufficient to describe the claimed genus.

Hence, Applicant has not, in fact, described nucleic acids with 90%, 93%, 94% or 95% identity to SEQ ID NO:1 and that encode a protein pesticidal for at least one pest belonging to the order Coleoptera within the full scope of the claims, and the specification fails to provide an adequate written description of the claimed invention.

Therefore, given the lack of written description in the specification with regard to the structural and physical characteristics of the claimed compositions, it is not clear that Applicant was in possession of the genus claimed at the time this application was filed.

#### **(10) Response to Argument**

a. Claims 1-3, 9-12, 17-19, 38, 43, 46, 49, 52 and 55-64 do not meet the full enablement requirement of 35 U.S.C. 112, first paragraph.

*The claimed invention is not fully taught in the specification.*

Appellant argues that sequences that fall within the scope of the claims, guidance for making alterations, methods for assaying, a discussion of Cry-8-like endotoxins, guidance for determining percent identity and specific mutations that fall within the scope of the claims (Brief, pg 6-7).

However, the guidance is insufficient, given the scope of the claims.

Limiting the percent identity of the claimed nucleic acid and requiring a function do not teach which amino acid substitutions may be made in the proteins. The guidance on pg 17-18 and 33-37 merely discusses fragment size, percent identity, and calculation of percent identity. However, guidance for determining percent identity does not teach which amino acid substitutions are permissible. The guidance fails to sufficiently teach which 362 amino acid substitutions to make in SEQ ID NO:2, given the unpredictability in making amino acid substitutions in *Cry* proteins.

Appellant argues that they teach multiple truncated variants of SEQ ID NO:1, as shown in the Brief's Table, and the variants have 38-92% identity over the full-length of SEQ ID NO:1, as calculated on a program available over a web-site; a copy of that webpage is not in the case (Brief, pg 7-8).

However, the specification does not teach nucleic acid within the full scope of the claims.

First, SEQ ID NO:27 is the genomic sequence. It encodes SEQ ID NO:2, and, as indicated by the rejection, nucleic acids encoding SEQ ID NO:2 are enabled. SEQ ID NO:9 is maize-optimized sequence encoding the truncation, SEQ ID NO:10. Although not claimed, nucleic acids encoding SEQ ID NO:10 are enabled.

Art Unit: 1638

Second, it is worth noting that the nucleic acids themselves are not pesticidal for Coleopterans; the proteins they encode are. These proteins are summarized in Table A below:

TABLE A.

Protein	Corresponds to	Same sequence as	Encoded by
SEQ ID NO:2	full-length Cry1218-1 protein		SEQ ID NO:1, SEQ ID NO:27
SEQ ID NO:6	amino acids 1-667 of SEQ ID NO:2		SEQ ID NO:5
SEQ ID NO:16	amino acids 1-669 of SEQ ID NO:2	SEQ ID NO:10	SEQ ID NO:15, SEQ ID NO:9
SEQ ID NO:12	amino acids 1-669 of SEQ ID NO:2 with NGSR inserted between amino acids 164 and 165		SEQ ID NO:11
SEQ ID NO:22	amino acids 1-669 of SEQ ID NO:2 with LKMS inserted between amino acids 164 and 165		SEQ ID NO:21, SEQ ID NO:39
SEQ ID NO:24	amino acids 1-669 of SEQ ID NO:2 with amino acids 164-166 replaced with LKMS	SEQ ID NO:44	SEQ ID NO:23
SEQ ID NO:20	amino acids 48-663 of SEQ ID NO:2		SEQ ID NO:19
SEQ ID NO:30	amino acids 48-663 of SEQ ID NO:2 with NGSR inserted between amino acids 164 and 165		SEQ ID NO:29
SEQ ID NO:32	amino acids 48-663 of SEQ ID NO:2 with LKMS inserted between amino acids 164 and 165	SEQ ID NO:42	
SEQ ID NO:34	amino acids 48-663 of SEQ ID NO:2 with amino acids 164-166 replaced with LKMS		SEQ ID NO:33
SEQ ID NO:4	full-length Cry1218-2 protein		SEQ ID NO:3 SEQ ID NO:28
SEQ ID NO:8	amino acids 1-667 of SEQ ID NO:4		SEQ ID NO:7
SEQ ID NO:18	amino acids 1-673 of SEQ ID NO:4		SEQ ID NO:17

As can be seen, there is little diversity in the sequence of the proteins taught in the specification. All that is taught are SEQ ID NO:s 2 and 4, and two truncations of SEQ ID NO:2 and 4 and variants of the SEQ ID NO:2 truncations with several small mutations at one particular site in the protein. One of ordinary skill in the art knows that it is only the N-terminal half the large *Cry* proteins that is the actual toxin (see for example, de Maagd, pg 4369, left column, paragraph 1). Further, it is well-known that the full-length protein is difficult to express in plants, and expression of the truncated protein is sufficient to confer pesticidal activity to the plant, (see, for example, *Adang v. Fischhoff* 62 USPQ2d 1504). Thus, the truncated proteins are obvious from the teaching of SEQ ID NO:2.

Art Unit: 1638

One of skill in the art would not consider a truncated protein as having 56% identity to the full length protein, but would only look at it as a truncated protein. The local match similarity between SEQ ID NO:15 and the first half of SEQ ID NO:1 is 100%. This can be represented as in the following 20 amino acid long “protein” in which the Original amino acids are represented by “O”:

Original:	OOOOOOOOOOOOOOOOOOOOOO
Truncated with 50% query match similarity	OOOOOOOOOO

One of skill in the art would understand a protein with 50% identity to encompass the following types of proteins, where in the following “S” represents any amino acid other than the original:

Original:	OOOOOOOOOOOOOOOOOOOOOO
Protein with 50% identity	SOSOSOSOSOSOSOSSSOSO
Protein with 50% identity	SOOSOSOSOSOSOSSOSSOOS
Protein with 50% identity	OSOSOSOSOSOSOSOSOSSO
Protein with 50% identity	OOSOSOSOSOSOSOSOSOSO

Thus, SEQ ID NO:16 does not teach a protein with 56% identity to SEQ ID NO:2, and SEQ ID NO:15, the nucleic acid that encodes SEQ ID NO:16, does not teach a nucleic acid with 55% identity to SEQ ID NO:1.

Similarly, SEQ ID NO:20 does not teach a protein with 52% identity to SEQ ID NO:2 - it consists of amino acids 48-663 of SEQ ID NO:2 - and SEQ ID NO:21, the nucleic acid that encodes SEQ ID NO:20, does not teach a nucleic acid with 55% identity to SEQ ID NO:1. Further it's identification of SEQ ID NO:20 as having 92% local identity to amino acids 1-699 of SEQ ID No:2 is equally misleading, as SEQ ID NO:20 only spans amino acids 48-663 of SEQ ID NO:2.

Art Unit: 1638

That the specification provides guidance in making nucleic acids encoding proteins with truncations at amino acid 663, 699 and 667, eliminating the C-terminus, and truncations off a portion of the N-terminus (in which the first 47 amino acids are eliminated) is not disputed. However, this is not anywhere near the full scope of the genus of claimed nucleic acids.

Further, even if one were to consider SEQ ID NO:20 and 16 as teaching proteins with 52% and 56% identity to SEQ ID NO:2, they would not teach how to make proteins with 362 amino acid substitutions over the entire length of SEQ ID NO:2.

Third, the 4 amino acid long substitutions/insertions at amino acid 164 of SEQ ID NO:2 are a teaching of variants, but these variants do not teach the full scope of the claimed variants. One of skill in the art would not consider these sequences as teaching which 181, 217, 253 or 362 amino acids to substitute in SEQ ID NO:2.

Fourth, SEQ ID NO:3 differs from SEQ ID NO:1 primarily in a 400 nucleotide long region (see search results; SEQ ID NO:2 and 4 differ by only 16 amino acids over amino acids 1-514 of SEQ ID NO:2, and are identical over amino acids 655-1206 of SEQ ID NO:2). Because the vast majority of substitutions are localized to such a small area, the protein it encodes, SEQ ID NO:4, has only about 132 amino acid substitutions relative to SEQ ID NO:2. Thus, SEQ ID NO:3 cannot provide all the necessary guidance for making nucleic acids with 90% identity to SEQ ID NO:1 and encoding a protein with 362 amino acid substitutions relative to SEQ ID NO:2, as encompassed by the full scope of the claims.

In summary, the specification fails to teach how to make nucleic acids within the full scope of the claims.

Art Unit: 1638

Appellant argues that SEQ ID NO:19 comprises only 1860 nucleotides of the 3621 nucleotides of SEQ ID NO:1, but it still retains activity, showing that it is likely that altering one or more of the other 1761 nucleotides of SEQ ID NO:1 would not disrupt function; furthermore, SEQ ID NO:9 encodes the same protein as SEQ ID NO:19 but has maize optimized codons, and it shares 38% global identity to SEQ ID NO:1 and 68% local identity (Brief, pg 8).

However, one of skill in the art would not consider a truncated protein as providing guidance for making amino acid substitutions in SEQ ID NO:2. Substitutions in the latter half of the protein affect the folding of the entire protein, and cannot be made willy-nilly just because a protein can be truncated.

All the studies on Cry protein structure and *Cry* protein amino acid substitutions look only at the N-terminal half of the proteins (See Li et al, Tounsi et al, de Maagd et al, and Angsuthanasombat et al). There is no guidance in the art or the specification for making amino acid substitutions in the C-terminal half; because no guidance is provided, one would have to use trial and error experimentation to make nucleic acids within the full scope of the claims. Multiple amino acid substitutions within the C-terminal half of the *Cry* protein would alter overall protein structure, affecting its stability of the protein and/or availability of the protease site to insect enzymes, thus rendering the protein non-toxic.

Further, the claims encompass nucleic acids that encode Coleopteran pesticides that have substitutions over the entire length of SEQ ID NO:2, not just substitutions in one region. The specification does not teach how to make such nucleic acids.



*The teachings in the specification are not sufficient for one of skill in the art to make and use the claimed invention*

Appellant argues that the examiner overlooks the guidance in the specification for making nucleic acids with 90%, 93%, 94% or 95% identity to SEQ ID NO:1; guidance is provided on pg 8 and 29 and examples 4, 6 and 7 (Brief, pg 8-9).

However, the guidance provided in the specification is not sufficient to teach how to make nucleic acids within the full scope of the claims.

Pg 8 of the specification defines terms like “protein”, “amino acid”, “isolated”, and “pesticidal activity”, but does not provide any guidance for making nucleic acids encoding Coleopteran pesticidal proteins with 362 amino acid substitutions relative to SEQ ID NO:2.

The specification, on pg 29, suggests using GenBank U04365 as the other nucleic acid in shuffling to create the claimed nucleic acids; however, GenBank U04365 encodes a protein with 79.8% identity to the instant SEQ ID NO:2. Thus, this sequence cannot be used as guidance for nucleic acids with 90% identity to SEQ ID NO:1 and that encode proteins with 70% identity to SEQ ID NO:2, as encompassed by the full scope of the claims.

Furthermore, SEQ ID NO:3 of Michaels et al is pesticidal for *Cyclocephala borealis* (northern masked chafer) and *Popillia japonica* (Japanese beetle), whereas the instant SEQ ID NO:1 is pesticidal to southern corn rootworm. Given the teachings of Tounsi et al, de Maagd et al, and Angsuthanasombat et al, one of skill in the art could not even predict for which pests the protein encoded by nucleic acids that have 90% identity to SEQ ID NO:1 would be toxic, and the specification does not teach which amino acid substitutions to make to retain proper pesticidal activity. The claims require that the protein be pesticidal for Coleopterans; the

Art Unit: 1638

specification does not teach the full scope of substitutions that would retain that activity.

Examples 4, 6 and 7 teach making the truncations and the two 4 amino acid substitutions presented in Table 1 of the Reply Brief. Why these examples do not teach nucleic acids within the full scope of the claims is discussed above.

Further, the specification on pg 3, lines 4-8, even acknowledges the unpredictability in making amino acid substitutions in Cry proteins:

Although numerous investigators have attempted to make mutant endotoxin proteins with improved insecticidal activity, few have succeeded. In fact, the majority of genetically engineered *B. thuringiensis* toxins that have been reported in the literature report endotoxin activity that is no better than that of the wild-type protein, and in many cases, the activity is decreased or destroyed altogether.

Appellant argues that the amount of experimentation required is not undue - claim 9 requires the steps required of claim 1 plus the transformation of a plant; because plant transformation is not undue, claim 9 requires no undue experimentation. Applicant makes a similar analysis for claim 17 (Brief, pg 9)

However, making nucleic acids within the full scope of the claims requires undue experimentation.

Making the nucleic acids would require undue experimentation because the specification does not provide sufficient guidance as to which 362 amino acid substitutions can be made in SEQ ID NO:2. Thus, one would need to randomly make nucleic acids encoding proteins with 362 amino acid substitutions and test them. Because the lack of guidance in the specification means this would require trial and error experimentation, because of the likelihood of protein inactivation (see Guo et al, pg 9209, right column, paragraph 2), and because of the unpredictability of amino acid interactions in *Cry* proteins (de Maagd et al, 1999, pg 4369, column 1, paragraph 1), this experimentation would be undue.

Art Unit: 1638

Appellant argues that SEQ ID NO:3 has 92% identity to SEQ ID NO:1, providing guidance for which residues can be mutated, and the maize-optimized SEQ ID NO:9, which has 38% global identity to SEQ ID NO:12, provides further guidance (Brief, pg 9-10).

However, SEQ ID NO:9 encodes amino acids 1-669 of SEQ ID NO:2; there are no amino acid substitutions in the encoded protein. The protein SEQ ID NO:3 encodes, SEQ ID NO:4, has only about 132 amino acid substitutions relative to SEQ ID NO:2.

Thus, none of SEQ ID NO: 3, SEQ ID NO:9 or the specification provide sufficient guidance for making nucleic acids with 90% identity to SEQ ID NO:1 and encoding a protein with 362 amino acid substitutions relative to SEQ ID NO:2.

Appellant argues that shuffling could be used to make the claimed nucleic acids, as was customary at the time of filing, citing US 5,837,458, Minshull et al and Christians et al (Brief, pg 10).

However, with respect to using DNA shuffling, the specification, on pg 29, suggests using GenBank U04365, which is identical to SEQ ID NO:3 of Michaels et al (1996, US Patent 5,554,534), as the other nucleic acid in shuffling; however, this sequence encodes a protein with 79.8% identity to the instant SEQ ID NO:2. Thus, such a nucleic acid could not be used to generate a nucleic acid that encodes a Coleopteran pesticidal protein with 70% identity to SEQ ID NO:2. Furthermore, Christians et al did not produce proteins with only 70% identity to the starting protein (see paragraph spanning pg 260-261).

Minshull et al teaches that a population should be used as the starting material (pg 284, right column, paragraph 4); the specification does not teach such a population. Minshull et al also teaches that the activities of chimeric enzymes are not predictable simply by comparing

Art Unit: 1638

those of the parent enzymes (paragraph spanning pg 288-289); thus, even if the population of starting materials has been provided, making nucleic acids that encode proteins pesticidal to coleopterans and have substitutions within the full scope of the claims is not predictable.

Thus, the teachings of '458, Minshull et al and Christians et al are insufficient to provide guidance for making the claimed nucleic acids, and Minshull et al argues that doing so would be unpredictable.

Appellant argues that at the time of filing it was routine to mutate amino acids in a protein then test for activity, citing Lazar et al, Hill et al, Tounsi et al, de Maagd et al, and Angsuthanasombat et al; these references show it is possible to make the claimed nucleic acids (Brief, pg 10-11).

However, although point mutations and substitutions of a few amino acids have been made in *Cry* proteins, no one has substituted 362, 253, 217 or 181 amino acids of a *Cry* protein, as encompassed the claimed nucleic acids. Angsuthanasombat, de Maagd et al, and Tounsi et al show that interactions between amino acids in *Cry* proteins is much more complex than can be predicted from guidance in the specification or the art.

de Maagd were surprised by many of their results (pg 4373, right column, paragraphs 2-3), showing that the residues have complex and unpredictable interactions with one another or with the insect receptor.

The amino acids critical to SEQ ID NO:2 activity are not taught, nor can they be predicted from the art.

Appellant argues that the specification teaches *Cry* proteins have 5 conserved blocks and 3 domains, and because the truncated protein retains activity, the deleted region would tolerate changes made using the guidance in the specification (Brief, pg 11).

However, the claims are not limited to changes made only in the deleted region, of such changes even can be made. The 5 conserved blocks and 3 domains in the N-terminal half of the protein (the non-deleted region) are not also sufficient to teach which 362 amino acid substitutions to make over the full length of the protein, as encompassed by the full scope of the claims.

Appellant argues that because assays for determining if the protein retains activity were disclosed, one could make and test the modified nucleic acids, and nothing more is required to enable the claims (Brief, pg 11-12).

However, the teachings for how to make the claimed nucleic acids are inadequate for the full scope of those nucleic acids. Thus, the making and testing cycle could be repeated ad infinitum without success.

Appellant argues that the Declaration of Dr. Abad stated that the procedures in examples 4, 6 and 7 were routine, and Lazar et al, Hill et al, Tounsi et al, de Maagd et al, and Angsuthanasombat et al support this (Brief, pg 12).

However, the procedures in examples 4, 6 and 7 only teach two truncations and two four amino acid insertions/substitution at the same site in the protein. These examples and the prior art only teach how to make a very small subset of the nucleic acids encompassed by the claims. None of these teach how to make a nucleic acid encoding a Coleopteran pesticidal protein with

Art Unit: 1638

362 amino acid substitutions distributed over the full length of SEQ ID NO:2, as encompassed by the claims.

*The reasoning provided by the Office is well-founded.*

Appellant argues that guidance is provided for which sequence alterations may be made, and the claims require both a structural component and a functional one; Appellant again refers to Table 1 of the Brief (Brief, pg 12-13).

However, Table 1 only teaches a very small subset of the claimed nucleic acids and does not provide guidance for making the full scope of the claimed nucleic acids, as discussed above. Further, a requirement that the nucleic acid has both a particular structure and a particular function does not teach which structure confers the claimed function.

Appellant argues that the analysis that one must make and test all possible combinations is incorrect, because, citing *In re Borkowski*, is it improper determine an appellant's claims are broader than they really are (Brief, pg 13).

However, Appellant's claims actually do encompass nucleic acids encoding Coleopteran pesticidal proteins with 362 amino acid substitutions distributed over the entire length of SEQ ID NO:2. Appellant has not denied that such nucleic acids are encompassed by their claims. Thus, *In re Borkowski* does not apply to the instant case.

Appellant argues that the specification provides guidance for making modifications, describes the domains of *Cry* proteins, provides insights into where modifications may be made, and teaches assays and variants (Brief, pg 13-14).

However, the guidance and insights are not sufficient for making nucleic acids encoding Coleopteran pesticidal proteins with 362 amino acid substitutions distributed over the entire length of SEQ ID NO:2. The variants and teaching of assays do not make up for this lack.

Appellant argues that the examiner overlooks the guidance in Li et al, as cited on pg 25 of the specification (Brief, pg 14).

However, Li et al only provides guidance for making truncations and insertion of chymotrypsin cleavage sites; Li et al do not provide guidance for making 181, 217, 253 or 362 amino acid substitutions in a 1206 amino acid protein. Further, de Maagd et al, 1999, teach that the crystal structure of Cry1C only allows for limited prediction of the structure of the closely related Cry1Aa (pg 4373, right column, paragraph 4). Thus, Li et al would provide even less guidance for making a Cry-8 like protein, which is even less related to Cry3A than Cry1C is to Cry1Aa.

Further, the instant inventors did not use Li et al to make 181, 217, 253 or 362 amino acid substitutions in a 1206 amino acid protein to create a Coleopteran pesticidal protein.

Appellant argues that SEQ ID NO:3 is a natural variant of SEQ ID NO:1 (Brief, pg 15).

However, SEQ ID NO:3 does not encode a protein that has 362 amino acid substitutions relative to SEQ ID NO:2 - it encodes a protein that has only about 132 amino acid substitutions relative to SEQ ID NO:2. Thus, it does not provide guidance for making nucleic acids within the full scope of the claims.

Appellant argues that SEQ ID NO:19 has 52% global identity to SEQ ID NO:1, enabling claims to nucleic acids with 90% identity to SEQ ID NO:1 (Brief, pg 15).

Art Unit: 1638

However, SEQ ID NO:19 encodes a protein the consists of the first 669 amino acids of SEQ ID NO:2. Such truncated sequences do not provide guidance for how to make nucleic acids that encode Coleopteran pesticidal proteins with 362 amino acid substitutions relative to SEQ ID NO:2.

Appellant argues that the truncated sequences establish that a region can be deleted to produce a protein that retains activity; one of skill in the art would recognize that this region would likely tolerate modifications, as well as which regions can be deleted (Brief, pg 15-16).

However, one of skill in the art would recognize that multiple amino acid substitutions within the C-terminal half of the Cry protein would alter overall protein structure, affecting its stability of the protein and/or availability of the protease site to insect enzymes, thus rendering the protein non-toxic. Deletion is not the same thing as amino acid substitution. Furthermore, the claims are not limited to nucleic acids that encode proteins that have only amino acid substitutions in the C-terminal half of SEQ ID NO:2, but encompass nucleic acids that encode Coleopteran pesticidal proteins with 362 amino acid substitutions distributed over the entire length of SEQ ID NO:2, as well as nucleic acids that encode Coleopteran pesticidal proteins with 362 amino acid substitutions in only the N-terminal half of SEQ ID NO:2. The specification does not teach how to make any of these.

Appellant argues that the claims do not require a deletion or substitution, only that the nucleic acids have the recited percent identity (Brief, pg 16).

However, the claims encompass nucleic acids that encode Coleopteran pesticidal proteins with 362 amino acid substitutions relative to SEQ ID NO:2. They are not limited to nucleic acids that encode SEQ ID NO:2 or its truncations, SEQ ID NOs:16 and 20.



Art Unit: 1638

Appellant argues that the requirement that Applicant make and test every possible substitution is improper, and many changes can be made that do not change the amino acid sequence of the encoded protein (Brief, pg 16).

However, making and testing every possible substitution is not required. One must practice trial and error experimentation, however, because the guidance in the specification is so insufficient. Practicing trial and error experimentation may well mean that an impossibly large number of possible substitutions must be tested. The numbers of possible single amino acid substitutions is presented to illustrate the scale of the trial and error experimentation required.

Appellant argues that SEQ ID NO:3 provides support for substitutions that can be made (Brief, pg 16).

However, SEQ ID NO:3 does not provides support for substitutions within the full scope of the claims. SEQ ID NO:3 does not encode a protein that has 362 amino acids substitutions relative to SEQ ID NO:2 - it encodes a protein that has only about 132 amino acid substitutions relative to SEQ ID NO:2. Thus, it does not provide guidance for making nucleic acids within the full scope of the claims.

*The experimentation required is undue.*

Appellant argues that the examiner has not established that the experimentation required is undue and has ignored the guidance in the specification (Brief, pg 17-18).

However, the guidance in the specification is not sufficient for making nucleic acids encoding Coleopteran pesticidal proteins with 362 amino acid substitutions distributed over the

Art Unit: 1638

entire length of SEQ ID NO:2, as encompassed by the full scope of claims. Thus, making these nucleic acids, if it is even possible to do so, would require undue experimentation.

See *Amgen Inc. v. Chugai Pharmaceutical Co. Ltd.*, 18 USPQ 2d 1016 at page 1027:

... despite extensive statements in the specification concerning all the analogs of the EPO gene that can be made, there is little enabling disclosure of particular analogs and how to make them. Details for preparing only a few EPO analog genes are disclosed. Amgen argues that this is sufficient to support its claims; we disagree. This "disclosure" might well justify a generic claim encompassing these and similar analogs, but it represents inadequate support for Amgen's desire to claim all EPO gene analogs. There may be many other genetic sequences that code for EPO-Type products. Amgen has told how to make and use only a few of them and is therefore not entitled to claim all of them.

*The cited references support the rejection.*

Appellant argues that Lazar is drawn to transforming growth factor alpha, a small mammalian peptide unrelated to *Cry* proteins and thus irrelevant to the claims; it would be unsurprising to one of skill in the art that changes in conserved amino acids would lead to loss of function and the purpose of Lazar was to study the primary structure of the protein (Brief, pg 18-19).

However, Lazar et al teach the unpredictability in making amino acid substitutions in proteins, and this unpredictability is applicable to large non-mammalian proteins. Further, Lazar et al only made single amino acid substitutions. The claims encompass nucleic acids that having 90% identity to SEQ ID NO:1 and encoding Coleopteran pesticidal proteins with 362 amino acid substitutions. Guo et al teaches that increasing the number of substitutions above single amino acid changes additively increases the probability that the protein will be inactivated (pg 9209, right column, paragraph 2). Thus, without further guidance, one of skill in the art could not make this many substitutions and still produce functional proteins.

Appellant argues that Hill is drawn to ADP-glucose pyrophosphorylase, proteins unrelated to *Cry* proteins and thus irrelevant to the claims; it would be unsurprising to one of

Art Unit: 1638

skill in the art that changes in conserved amino acids would lead to loss of function, and that Hill shows strictly conserved residues whose functions are essential (Brief, pg 19).

However, Hill et al teach the unpredictability in making amino acid substitutions in proteins with a number of homologs, and this unpredictability is applicable to other proteins. Hill et al made single amino acid substitutions, and found unpredictably in those; making nucleic acids that having 90% identity to SEQ ID NO:1 and encoding Coleopteran pesticidal proteins with 362 amino acid substitutions would be much more unpredictable. Guo et al teaches that increasing the number of substitutions above single amino acid changes additively increases the probability that the protein will be inactivated (pg 9209, right column, paragraph 2). Thus, without further guidance, one of skill in the art could not make this many substitutions and still produce functional proteins.

Appellant argues that de Maagd teaches that much is known about *Cry* protein structure and that essential residues have been identified in insect-specific toxicity; this knowledge can be used to make substitutions in the claimed nucleic acids (Brief, pg 20).

However, de Maagd were surprised by many of the results (pg 4373, right column, paragraphs 2-3), showing that the residues have complex and unpredictable interactions with one another or with the insect receptor. Further, de Maagd et al teach that that the crystal structure of Cry1C only allows for limited prediction of the structure of Cry1Aa (pg 4373, right column, paragraph 4). The essential residues for insect-specific toxicity must be determined for each *Cry* protein.

Art Unit: 1638

Appellant argues that Tounsi shows that using alignments and domain information substitutions can be made in *Cry* proteins, and Angsuthanasombat show that mutations in regions known to be essential for toxicity effect toxicity (Brief, pg 20-21).

However, Tounsi and Angsuthanasombat show that single amino acid substitutions made using alignments and domain information were unpredictable. If single amino substitutions were that unpredictable, making 362 amino acid substitutions, including in regions of the protein where no domain information is known, would be even more unpredictable.

*The specification does not meet the standard for enablement.*

Appellant argues that that one would only need to make the claimed variants and assay them for activity using routine methods; thus the amount of experimentation is not undue; the plant transformation steps also do not require undue experimentation (Brief, pg 22).

However, while the plant transformation steps are relatively straightforward, making nucleic acids within the full scope of the claims would require undue experimentation, given the insufficient guidance in the specification.

Appellant argues that based on the guidance of exemplary nucleic acids and proteins and methods for assaying, one of skill in the art could choose among possible sequence modifications and test them for activity; thus the quantity of experimentation is not undue, citing nonprecedential Board decisions (Brief, pg 22).

However, the number of modifications encompassed by and required by the claims would require undue experimentation in making those nucleic acids. Furthermore, Federal Circuit Rule 47.6 prohibits the citation of nonprecedential opinions.

Appellant argues that just because some experimentation is required, it does not mean that the experimentation is undue (Brief, pg 23).

However, make nucleic acids with 90%, 93%, 94% or 95% identity to SEQ ID NO:1 and encoding Coleopteran pesticidal proteins with up to 362, 253, 217 or 181 amino acid substitutions relative to SEQ ID NO:2 would require undue experimentation, given that the specification does not teach how to make such nucleic acids.

*Claims 38, 43, 49, 55, 56, 58, 59, 63 and 64 are also not enabled.*

Appellant argues that these claims are narrower in scope and should be considered in view of the arguments above (Brief, pg 24).

However, the specification also does not teach how to make nucleic acids with 93%, 94% or 95% identity to SEQ ID NO:1 and encoding Coleopteran pesticidal proteins with up to 253, 217 or 181 amino acid substitutions relative to SEQ ID NO:2.

b. Claims 1-3, 9-12, 17-19, 38, 43, 46, 49, 52 and 55-64 do not meet the written description requirement of 35 U.S.C. 112, first paragraph.

*The claimed sequences are not adequately described in the specification.*

Appellant argues that the Table in the Brief shows sequences that have been modified but still retain the activity (Brief, pg 25).

However, the sequences in the Table do not describe the structure of nucleic acid within the full scope of the claims. No nucleic acid with 90% identity to SEQ ID NO:1 and that encodes a Coleopteran pesticidal protein with 70% identity to the original protein has been described.

Appellant argues that individual support for each species is not required, only that a representative number be described (Brief, pg 25).

However, a representative number of species encompassing the full scope of the claims have not been described. The specification has not described the structure of any nucleic acid with 90% identity to SEQ ID NO:1 and that encodes a Coleopteran pesticidal protein with 362 amino acid substitutions relative to SEQ ID NO:2, much less the full scope of such nucleic acids.

Appellant argues that the Written Description Guidelines, *Lilly* and *Enzo* state that written description requires a precise definition by structure, which is present in the 90% identity recitation (Brief, pg 26).

However, *Enzo* states: “the written description requirement would be met ... if the functional characteristics of [a genus of polypeptides] were coupled with a disclosed correlation between that function and a structure that is sufficiently known or disclosed” (emphasis added). The correlation between that function and a structure is not sufficiently known in *Cry* proteins as a whole, and the description and assays provided in the specification are insufficient.

Only a portion of the structural features have been described - the percent identity to SEQ ID NO:1. But because this includes nucleic acids in which the protein sequence has a very large number of amino acid substitutions, those amino acid substitutions that do not alter the function of the protein must be described. They are not. A representative number of nucleic acids that

Art Unit: 1638

have 90%, 93%, 94% or 95% identity to SEQ ID NO:1 and up to 362, 253, 217 or 181 amino acid substitutions, respectively, relative to the 1206 amino acid long SEQ ID NO:2 are not described.

Appellant argues that Example 14 of the Written Description Guidelines says that proteins with 95% identity to a given sequence identifier and that have a specified activity are described (Brief, pg 26-27).

However, the instant claims are not drawn to proteins with 95% identity to a given sequence identifier and that have a specified activity or to nucleic acids encoding such proteins. The claims encompass nucleic acids that encode proteins that have 70%, 79%, 82% and 85% identity to SEQ ID NO:2. Thus, the fact pattern in the instant case is different from that in Example 14 of the Written Description Guidelines.

*There is insufficient written description support in the specification.*

Appellant argues that the specification describes 14 variants with 38%-92% identity to SEQ ID NO:1 (Brief, pg 28-29).

However, these variants are very similar to one another. The full scope of nucleic acids encompassed by the claims are not described. For example, the specification does not describe the structure of any nucleic acid encoding a Coleopteran pesticidal protein with 362 amino acid substitutions distributed over the full length of SEQ ID NO:2.

*Lilly applies to the instant case.*

Art Unit: 1638

Appellant argues that *Invitrogen* indicates that that case is different from the fact pattern in *Lilly* and *Feirs*, and argue this would apply here (Brief, pg 29-30).

However, the fact pattern in *Invitrogen* is different from the instant case; the claims in *Invitrogen* were drawn to known proteins mutated so that one function is lost. In the instant case, the claims are drawn to proteins in which up to 30% of the amino acids are substituted but that retain the activity. The structural features of such nucleic acids are not described.

*Claims 38, 43, 49, 55, 56, 58, 59, 63 and 64 are also not described.*

Appellant argues that these claims are narrower in scope and should be considered in view of the arguments above (Brief, pg 30).

However, the specification also does not describe the structure of nucleic acids with 93%, 94% or 95% identity to SEQ ID NO:1 and that encode Coleopteran pesticidal proteins with up to, 253, 217 or 181 amino acid substitutions relative to SEQ ID NO:2.

#### **(11) Related Proceeding(s) Appendix**

No decision rendered by a court or the Board is identified by the examiner in the Related Appeals and Interferences section of this examiner's answer.

For the above reasons, it is believed that the rejections should be sustained.

Respectfully submitted,

Anne Kubelik

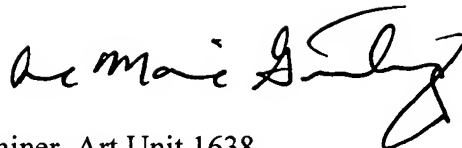
Primary Examiner



Art Unit: 1638

Conferees:

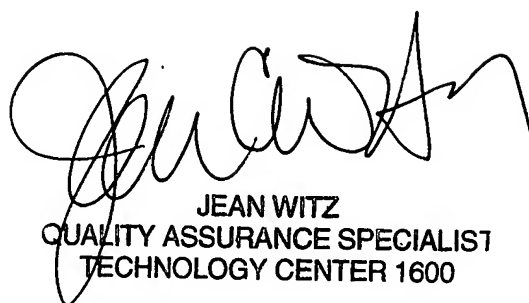
Anne Marie Grunberg



Supervisory Patent Examiner, Art Unit 1638

Jean Witz

Quality Assurance Specialist, TC 1600



JEAN WITZ  
QUALITY ASSURANCE SPECIALIST  
TECHNOLOGY CENTER 1600

# Appendix A

181 TQMPFRVTFVFPVFLVYVMAANLHLLKLDASI FCEENGSTTTTNNYDROMKLT 240  
181 TQMPFRVTFVFPVFLVYVMAANLHLLKLDASI FCEENGSTTTTNNYDROMKLT 240  
241 EYSDHCVRVETGLAKGTSKQWVDNPNREMTLAVLDVALPNDYDTPYMETKA 300  
241 EYSDHCVRVETGLAKGTSKQWVDNPNREMTLAVLDVALPNDYDTPYMETKA 300  
301 QLTREVTYDPLGAVNVSSIGSWYDKAPSGVIESVIRPPHVPDYITGLTYTQSRSS 360  
301 QLTREVTYDPLGAVNVSSIGSWYDKAPSGVIESVIRPPHVPDYITGLTYTQSRSS 360  
361 ARYIRHAGHQSIRHVRSGSNLQMGYNQNLHSTSTFTDNYDIYKLSKDAVLDD 420  
361 ARYIRHAGHQSIRHVRSGSNLQMGYNQNLHSTSTFTDNYDIYKLSKDAVLDD 420  
421 YPGYTIIPFGMEVEBPVFNQNLNTRKLTAKNPKVSKDI IASTRDSLELPPTSOPNYE 480  
421 YPGYTIIPFGMEVEBPVFNQNLNTRKLTAKNPKVSKDI IASTRDSLELPPTSOPNYE 480  
481 SYSHRLCHTSPATGNTTGLVPVFSWTHRSADLNTIYSKDIQIIPAVKMDNLPFPV 540  
481 SYSHRLCHTSPATGNTTGLVPVFSWTHRSADLNTIYSKDIQIIPAVKMDNLPFPV 540  
541 VKPGHGTGGLQVNRSGVGTFLARYGLALEKAGRVRLRYATDADIVLVNDQOI 600  
541 VKPGHGTGGLQVNRSGVGTFLARYGLALEKAGRVRLRYATDADIVLVNDQOI 600  
601 QMPKTNPGEDLTSTKTFKADAITTLATDSSSLKGNLGEDPNSTLSGIVVDRISPI 660  
601 QMPKTNPGEDLTSTKTFKADAITTLATDSSSLKGNLGEDPNSTLSGIVVDRISPI 660  
661 PVDETYEASODLEAKKAVNALPNTKOCARPCVTDYEVNQANLVECLSDDLVNEKRL 720  
661 PVDETYEASODLEAKKAVNALPNTKOCARPCVTDYEVNQANLVECLSDDLVNEKRL 720  
721 LFDVAREAGLSEANLQDDPDSINGENGWTA STGLEVEGDALPKGYELPGAREI 780  
721 LFDVAREAGLSEANLQDDPDSINGENGWTA STGLEVEGDALPKGYELPGAREI 780  
781 DTETYPYLYQVREBGLKPYTRVLRGPFVGSQGLEFTIRHQTNRIRVNPVDDLDPV 840  
781 DTETYPYLYQVREBGLKPYTRVLRGPFVGSQGLEFTIRHQTNRIRVNPVDDLDPV 840  
841 SPVNSDGSINRSECKYNSRLEVENRSGEABEFSIPIDAGEIDYENAGIWWGPKITDP 900  
841 SPVNSDGSINRSECKYNSRLEVENRSGEABEFSIPIDAGEIDYENAGIWWGPKITDP 900  
901 EGYATLGNLELVEBGLSGDALERLQREBQWKI QMTRRREBETDRYMASKQAVRLYAD 960  
901 EGYATLGNLELVEBGLSGDALERLQREBQWKI QMTRRREBETDRYMASKQAVRLYAD 960  
961 YQDQQLNPDEITDLTAQDLIQSI PVVYNEMFPEIPGNYTKETELDRLOQANSLYDQ 1020  
961 YQDQQLNPDEITDLTAQDLIQSI PVVYNEMFPEIPGNYTKETELDRLOQANSLYDQ 1020  
1021 RNAIPNGDFRNLGNNAATPGVEVQIINHTSVLVIPNDDEOVSOQFTVQNRVYLRVTA 1080  
1021 RNAIPNGDFRNLGNNAATPGVEVQIINHTSVLVIPNDDEOVSOQFTVQNRVYLRVTA 1080  
1081 RKEGVNGVYSIRDGNGTETLTPSASDYDNGTNTVSNNGYNTNNAYNQASSTNG 1140  
1081 RKEGVNGVYSIRDGNGTETLTPSASDYDNGTNTVSNNGYNTNNAYNQASSTNG 1140  
1141 YNANNMYNTQASNTNGYNTSVNDQGYITKTVPITPTOMKIEMSEBEGTPTTSSVE 1200  
1141 YNANNMYNTQASNTNGYNTSVNDQGYITKTVPITPTOMKIEMSEBEGTPTTSSVE 1200  
1201 LIVDVE 1206  
1201 LIVDVE 1206

RESULT 2  
US-10-032-717-4  
Sequence 4, Application US/10032717  
Patent No. US20020151709A1  
GENERAL INFORMATION:  
APPLICANT: Andre R. Abad  
APPLICANT: Nicholas B. Duck  
APPLICANT: Xiang Feng  
APPLICANT: Ronald D. Flannagan  
APPLICANT: Theodore W. Kahn  
APPLICANT: Lynn B. Sims  
TITLE OF INVENTION: Genes Encoding No. US20020151709A1e1 Proteins With  
Pesticidal Activity Against Coleopterans  
FILE REFERENCE: 35718/237005  
CURRENT APPLICATION NUMBER: US/10/032,717  
CURRENT FILING DATE: 2001-10-23  
PRIOR APPLICATION NUMBER: 60/242,838  
PRIOR FILING DATE: 2000-10-24  
NUMBER OF SEQ ID NOS: 48  
SOFTWARE: FastSeq for Windows Version 4.0  
SEQ ID NO 4  
LENGTH: 1210  
TYPE: PRT  
ORGANISM: Bacillus thuringiensis  
US-10-032-717-4  
Query Match 89.6%, Score 5673, DB 12, Length 1210;  
Best Local Similarity 89.2%, Pred. No. 0;  
Matches 1095, Conservative 33, Mismatches 61; Indels 38; Gaps 7;  
QY 1 MSPNNONEYEIIDATPSTVSNDNRYPPANEPTNALQNDYKDLKMSAGNASYPCSP 60  
DB 1 MSPNNONEYEIIDATPSTVSNDNRYPPANEPTNALQNDYKDLKMSAGNASYPCSP 60  
QY 61 EVLVSGQDAAKAADIVKGLSGIYCHVPFGVIGVSLYQLDILWPGSKSQWEIIPMEVE 120  
DB 61 EVLVSGQDAAKAADIVKGLSGIYCHVPFGVIGVSLYQLDILWPGSKSQWEIIPMEVE 120  
QY 121 ELINOKIABYARNKALSHLEGNNYQYLTALBEBENPNSGRALRDVNRPFILDSLP 160  
DB 121 ELINOKIABYARNKALSHLEGNNYQYLTALBEBENPNSGRALRDVNRPFILDSLP 160  
QY 181 TQMPFRVTFVFPVFLVYVMAANLHLLKLDASI FCEENGSTTTTNNYDROMKLT 240  
DB 181 TQMPFRVTFVFPVFLVYVMAANLHLLKLDASI FCEENGSTTTTNNYDROMKLT 240  
QY 241 EYSDHCVRVETGLAKGTSKQWVDNPNREMTLAVLDVALPNDYDTPYMETKA 300  
DB 241 EYSDHCVRVETGLAKGTSKQWVDNPNREMTLAVLDVALPNDYDTPYMETKA 300  
QY 301 QLTREVTYDPLGAVNVSSIGSWYDKAPSGVIESVIRPPHVPDYITGLTYTQSRSS 360  
DB 301 QLTREVTYDPLGAVNVSSIGSWYDKAPSGVIESVIRPPHVPDYITGLTYTQSRSS 360  
QY 361 ARYIRHAGHQSIRHVRSGSNLQMGYNQNLHSTSTFTDNYDIYKLSKDAVLDD 420  
DB 361 ARYIRHAGHQSIRHVRSGSNLQMGYNQNLHSTSTFTDNYDIYKLSKDAVLDD 420  
QY 421 YPGYTIIPFGMEVEBPVFNQNLNTRKLTAKNPKVSKDI IASTRDSLELPPTSOPNYE 480  
DB 421 YPGYTIIPFGMEVEBPVFNQNLNTRKLTAKNPKVSKDI IASTRDSLELPPTSOPNYE 480  
QY 481 SYSHRLCHTSPATGNTTGLVPVFSWTHRSADLNTIYSKDIQIIPAVKMDNLPFPV 540  
DB 481 SYSHRLCHTSPATGNTTGLVPVFSWTHRSADLNTIYSKDIQIIPAVKMDNLPFPV 540  
QY 539 ----PVVPGHGTGGLQVNRSGVGTFLARYGLALEKAGRVRLRYATDADIVLVNDQOI 593  
DB 541 GPNNTVSGPGFTGGGIKVRN-----GVIIISHMKVKSIDINKREYSMEIRYASANNTEP 595  
QY 594 HVNDQAIQW----PCTWNPGEHLTSTKTFKADAITTLATDSSSLKGNLGEDPNSTLS 649  
DB 596 YINPSEENVKSHAQTNRGEALTYNKNYA-TLPPIKFTT-----TEPPIILG 643

Instant seq ID 12012  
vs instant  
seq ID 12014

```

RESULT 3
US-10-032-717-10
  / Sequence 10, Application US/10032717
  / Patent No. US20020151709A1
  / GENERAL INFORMATION:
  / APPLICANT: Andre R. Abad
  / APPLICANT: Nicholas B. Duck
  / APPLICANT: Xiang Feng
  / APPLICANT: Ronald D. Flannegan
  / APPLICANT: Theodore W. Kahn
  / APPLICANT: Lynn B. Sims
  / TITLE OF INVENTION: Genes Encoding No. US20020151709A1e1 Proteins With
  / TITLE OF INVENTION: Pesticidal Activity Against Coleopterans
  / FILE REFERENCE: 3718/237005
  / CURRENT APPLICATION NUMBER: US/10/032,717
  / CURRENT FILING DATE: 2001-10-23
  / PRIOR APPLICATION NUMBER: 60/242,838
  / PRIOR FILING DATE: 2000-10-24
  / NUMBER OF SEQ ID NOS: 48
  / SOFTWARE: FastSeq for Windows Version 4.0
  / SEQ ID NO 10
  / LENGTH: 669
  / TYPE: PRT
  / ORGANISM: Artificial Sequence
  / FEATURE:
  / OTHER INFORMATION: Maize optimized Cry1218-1
US-10-032-717-10

Query Match          55.4%; Score 3341; DB 12; Length 669;
Best Local Similarity 100.0%; Pred. NO 5.6e-219;
Matches 669; Conservative 0; Mismatches 0; Indels 0; Gaps 0;

```

```

660
661
662
663
664
665
666
667
668
669
670
671
672
673
674
675
676
677
678
679
680
681
682
683
684
685
686
687
688
689
690
691
692
693
694
695
696
697
698
699
700
701
702
703
704
705
706
707
708
709
710
711
712
713
714
715
716
717
718
719
720
721
722
723
724
725
726
727
728
729
730
731
732
733
734
735
736
737
738
739
740
741
742
743
744
745
746
747
748
749
750
751
752
753
754
755
756
757
758
759
760
761
762
763
764
765
766
767
768
769
770
771
772
773
774
775
776
777
778
779
780
781
782
783
784
785
786
787
788
789
790
791
792
793
794
795
796
797
798
799
800
801
802
803
804
805
806
807
808
809
810
811
812
813
814
815
816
817
818
819
820
821
822
823
824
825
826
827
828
829
830
831
832
833
834
835
836
837
838
839
840
841
842
843
844
845
846
847
848
849
850
851
852
853
854
855
856
857
858
859
860
861
862
863
864
865
866
867
868
869
870
871
872
873
874
875
876
877
878
879
880
881
882
883
884
885
886
887
888
889
890
891
892
893
894
895
896
897
898
899
900
901
902
903
904
905
906
907
908
909
910
911
912
913
914
915
916
917
918
919
920
921
922
923
924
925
926
927
928
929
930
931
932
933
934
935
936
937
938
939
940
941
942
943
944
945
946
947
948
949
950
951
952
953
954
955
956
957
958
959
960
961
962
963
964
965
966
967
968
969
970
971
972
973
974
975
976
977
978
979
980
981
982
983
984
985
986
987
988
989
990
991
992
993
994
995
996
997
998
999
1000
1001
1002
1003
1004
1005
1006
1007
1008
1009
1010
1011
1012
1013
1014
1015
1016
1017
1018
1019
1020
1021
1022
1023
1024
1025
1026
1027
1028
1029
1030
1031
1032
1033
1034
1035
1036
1037
1038
1039
1040
1041
1042
1043
1044
1045
1046
1047
1048
1049
1050
1051
1052
1053
1054
1055
1056
1057
1058
1059
1060
1061
1062
1063
1064
1065
1066
1067
1068
1069
1070
1071
1072
1073
1074
1075
1076
1077
1078
1079
1080
1081
1082
1083
1084
1085
1086
1087
1088
1089
1090
1091
1092
1093
1094
1095
1096
1097
1098
1099
1100
1101
1102
1103
1104
1105
1106
1107
1108
1109
1110
1111
1112
1113
1114
1115
1116
1117
1118
1119
1120
1121
1122
1123
1124
1125
1126
1127
1128
1129
1130
1131
1132
1133
1134
1135
1136
1137
1138
1139
1140
1141
1142
1143
1144
1145
1146
1147
1148
1149
1150
1151
1152
1153
1154
1155
1156
1157
1158
1159
1160
1161
1162
1163
1164
1165
1166
1167
1168
1169
1170
1171
1172
1173
1174
1175
1176
1177
1178
1179
1180
1181
1182
1183
1184
1185
1186
1187
1188
1189
1190
1191
1192
1193
1194
1195
1196
1197
1198
1199
1200
1201
1202
1203
1204
1205
1206
1207
1208
1209
1210
1211
1212
1213
1214
1215
1216
1217
1218
1219
1220
1221
1222
1223
1224
1225
1226
1227
1228
1229
1230
1231
1232
1233
1234
1235
1236
1237
1238
1239
1240
1241
1242
1243
1244
1245
1246
1247
1248
1249
1250
1251
1252
1253
1254
1255
1256
1257
1258
1259
1260
1261
1262
1263
1264
1265
1266
1267
1268
1269
1270
1271
1272
1273
1274
1275
1276
1277
1278
1279
1280
1281
1282
1283
1284
1285
1286
1287
1288
1289
1290
1291
1292
1293
1294
1295
1296
1297
1298
1299
1300
1301
1302
1303
1304
1305
1306
1307
1308
1309
1310
1311
1312
1313
1314
1315
1316
1317
1318
1319
1320
1321
1322
1323
1324
1325
1326
1327
1328
1329
1330
1331
1332
1333
1334
1335
1336
1337
1338
1339
1340
1341
1342
1343
1344
1345
1346
1347
1348
1349
1350
1351
1352
1353
1354
1355
1356
1357
1358
1359
1360
1361
1362
1363
1364
1365
1366
1367
1368
1369
1370
1371
1372
1373
1374
1375
1376
1377
1378
1379
1380
1381
1382
1383
1384
1385
1386
1387
1388
1389
1390
1391
1392
1393
1394
1395
1396
1397
1398
1399
1400
1401
1402
1403
1404
1405
1406
1407
1408
1409
1410
1411
1412
1413
1414
1415
1416
1417
1418
1419
1420
1421
1422
1423
1424
1425
1426
1427
1428
1429
1430
1431
1432
1433
1434
1435
1436
1437
1438
1439
1440
1441
1442
1443
1444
1445
1446
1447
1448
1449
1450
1451
1452
1453
1454
1455
1456
1457
1458
1459
1460
1461
1462
1463
1464
1465
1466
1467
1468
1469
1470
1471
1472
1473
1474
1475
1476
1477
1478
1479
1480
1481
1482
1483
1484
1485
1486
1487
1488
1489
1490
1491
1492
1493
1494
1495
1496
1497
1498
1499
1500
1501
1502
1503
1504
1505
1506
1507
1508
1509
1510
1511
1512
1513
1514
1515
1516
1517
1518
1519
1520
1521
1522
1523
1524
1525
1526
1527
1528
1529
1530
1531
1532
1533
1534
1535
1536
1537
1538
1539
1540
1541
1542
1543
1544
1545
154
```

GenCore version 5.1.3  
Copyright (c) 1993 - 2003 Compugen Ltd.

OM nucleic - nucleic search, using sw model

Run on: January 7, 2003, 00:49:27 ; Search time 89 Seconds  
(without alignments)  
12477.265 Million cell updates/sec

Title: US-10-032-717-1  
Perfect score: 3621  
Sequence: 1 atgattccaaatacaaaa.....tgatttagacgtagtagtaa 3621

Scoring table: IDENTITY\_NUC  
Gapop 10.0, Gapext 1.0

Searched: 441362 seqs, 1533381 residues

Total number of hits satisfying chosen parameters: 882724

Minimum DB seq length: 0  
Maximum DB seq length: 2000000000

Post-processing: Minimum Match 0  
Maximum Match 100  
Listing first 45 summaries

Database : Issued Patents NA.\*  
1: /cgn2\_6/ptodata/1/ina/5A COMB.seq.\*  
2: /cgn2\_6/ptodata/1/ina/5B COMB.seq.\*  
3: /cgn2\_6/ptodata/1/ina/5A COMB.seq.\*  
4: /cgn2\_6/ptodata/1/ina/5B COMB.seq.\*  
5: /cgn2\_6/ptodata/1/ina/PTUS COMB.seq.\*  
6: /cgn2\_6/ptodata/1/ina/backfiles1.seq.\*

Pred. No. is the number of results predicted by chance to have a score greater than or equal to the score of the result being printed, and is derived by analysis of the total score distribution.

SUMMARIES

Result No.	Score	Query Match	Length	ID	Description
1	2565.2	70.8	3507	1	US-08-315-468-3
2	1812.2	50.0	3471	1	US-07-876-280-29
3	1812.2	50.0	3471	1	US-07-812-180A-1
4	1812.2	50.0	3471	1	US-08-315-468-1
5	1812.2	50.0	3471	4	US-07-941-650A-1
6	1180	32.6	3797	1	US-07-915-203-1
7	1180	32.6	3797	1	US-08-272-887-1
8	1180	32.6	3797	1	US-08-789-449-1
9	893.4	24.4	4344	2	US-08-532-547-4
10	893.4	24.4	4344	2	US-08-379-658B-4
11	893.4	24.4	4344	3	US-08-455-818-4
12	893.4	24.4	4344	3	US-09-019-809-4
13	893.4	24.4	4344	4	US-09-471-177-4
14	882.8	24.4	3471	4	US-09-002-285-73
15	848.6	23.4	3759	1	US-08-542-921-1
16	848.6	23.4	3759	2	US-08-880-685-1
17	848.6	23.4	3759	2	US-08-880-684-1
18	822.2	22.7	3453	4	US-09-002-285-75
19	734.2	20.3	3411	8	US-09-002-285-77
20	733.6	20.3	3411	1	US-07-973-320-3
21	731.4	20.2	3414	1	US-07-973-320-1
22	678	18.7	3934	1	US-08-100-709-3
23	678	18.7	3934	1	US-08-176-865-3
24	678	18.7	3934	1	US-08-474-038-3
25	678	18.7	3934	2	US-08-779-046-3
26	678	18.7	3934	2	US-08-881-340-3
27	673.6	18.6	4074	1	US-08-377-690-1

28	659.2	18.2	3684	1	US-08-448-170-7
29	659.2	18.2	3684	3	US-08-961-803-5
30	618.6	17.1	3567	6	5188960-5
31	615.4	17.0	3567	2	US-08-980-071-5
32	615.4	17.0	3567	2	US-08-980-071-58
33	615.4	17.0	3567	2	US-08-757-536-5
34	615.4	17.0	3567	3	US-09-314-093-5
35	615.4	17.0	3567	3	US-09-314-093-58
36	615.4	17.0	3567	3	US-09-250-848-5
37	615.4	17.0	3567	4	US-09-251-885-5
38	615.4	17.0	3567	4	US-09-337-635-5
39	615.4	17.0	3567	4	US-09-337-635-58
40	615.4	17.0	3567	4	US-09-337-280-5
41	615.4	17.0	3567	4	US-09-337-280-58
42	613.8	17.0	3567	1	US-08-602-737-1
43	613.8	17.0	3567	2	US-08-980-071-1
44	613.8	17.0	3567	2	US-08-980-071-9
45	613.8	17.0	3567	2	US-08-980-071-11

ALIGNMENTS

Instant SEQ ID NO: 1 vs SEQ ID NO: 3  
of Michaels  
et al

RESULT 1  
US-08-315-468-3  
; Sequence 3, Application US/08315468  
; Patent No. 5554534  
; GENERAL INFORMATION:  
; APPLICANT: Michaels, Tracy Ellis  
; APPLICANT: Poncerrada, Luis  
; APPLICANT: Narva, Kenneth E.  
; TITLE OF INVENTION: Process for Controlling Scarab Pests  
; TITLE OF INVENTION: With Bacillus thuringiensis Isolates  
; NUMBER OF SEQUENCES: 6  
; CORRESPONDENCE ADDRESS:  
; ADDRESSEE: David R. Salivanchik  
; STREET: 2421 N.W. 41st Street, Suite A-1  
; CITY: Gainesville  
; STATE: FL  
; COUNTRY: USA  
; ZIP: 32606  
; COMPUTER READABLE FORM:  
; MEDIUM TYPE: Floppy disk  
; OPERATING SYSTEM: PC-DOS/MS-DOS  
; SOFTWARE: PatentIn Release #1.0, Version #1.25  
; CURRENT APPLICATION NUMBER: US/08/315,468  
; FILING DATE:  
; CLASSIFICATION: 424  
; PRIOR APPLICATION DATA:  
; APPLICATION NUMBER: US/08/014,941  
; FILING DATE: 01 FEB 1993  
; APPLICATION NUMBER: 07/828,430  
; FILING DATE: 30-JAN-1992  
; PRIOR APPLICATION DATA:  
; APPLICATION NUMBER: 07/808,316  
; FILING DATE: 16-DEC-1991  
; ATTORNEY/AGENT INFORMATION:  
; NAME: Salivanchik, David R.  
; REGISTRATION NUMBER: 31,794  
; REFERENCE/DOCKET NUMBER: MA73.C2  
; TELECOMMUNICATION INFORMATION:  
; TELEPHONE: 904-375-8100  
; TELEFAX: 904-372-5800  
; INFORMATION FOR SEQ ID NO: 3:  
; SEQUENCE CHARACTERISTICS:  
; LENGTH: 3507 base pairs  
; TYPE: nucleic acid  
; STRANDEDNESS: double  
; TOPOLOGY: linear  
; MOLECULE TYPE: DNA (genomic)  
; HYPOTHETICAL: NO

ANTI-SENSE: NO  
ORIGINAL SOURCE: *Bacillus thuringiensis*  
ORGANISM: *Bacillus thuringiensis*  
STRAIN: Kumamotoensis  
INDIVIDUAL ISOLATE: 50C  
IMMEDIATE SOURCE:  
LIBRARY: LambdaGEN-11(tm) library of L. Foncecrrada  
CLONE: 50C(b)  
US-08-315-468-3

Query Match 70.8%; Score 2565.2; DB 1; Length 3507;  
Best Local Similarity 85.1%; Pred. NO. 0;  
Matches 2906; Conservative 0; Mismatches 493; Indels 15;

Qy	1	ATGAGTCCAAATTAATCAAAATGAATATGAATATATAGATGGACACCTTCTACTCTCGTA	60
Db	1	ATGAGTCCAAATTAATCAAAATGAATATGAATATATAGATGGACACCTTCTACTCTGTA	60
Qy	61	TCCAATGATTTCTAAACAGATACCCCTTTTGGGAAATGAGCCAAACAAATCGCTACAAAATATG	120
Db	61	TCCAATGATTTCTAAACAGATACCCCTTTTGGGAAATGAGCCAAACAAATCGCTACAAAATATG	120
Qy	121	GATTATAAAGATTATTTAAAAATGCTGCGGGAAATGCTAGTCAATACCCCTGGTTTCACTT	180
Db	121	GATTATAAAGATTATTTAAAAATGCTGCGGGAAATGTTAGTCAATACCCCTGGTTTCACTT	180
Qy	181	GAAGTACTGCTTAGCGGACAGATGCTAGGCGCGCAATGATATAGTAGGTAAATTA	240
Db	181	GAGGTATTTCTTAGCGGACAGATGCTAGGCGCGCAATGATATAGTAGGTAAATTA	240
Qy	241	CTATCAGGTTTAGGGTCCCAATTTGTTGGCCGATAGTGAAGTCTTTATATCTCAACTTAT	300
Db	241	CTAAACAGGTTTAGGGGTTTCCATTTGTTGGGCCGATAGTGAAGTCTTTATATCTCAACTTAT	300
Qy	301	GATATTCTGTGGGCTTTCAGGGGAAAAGAGTCAATGGGAAATTTTATATGGAACAAGTAGAA	360
Db	301	GATATTCTGTGGGCTTTCAAAAACAAAAGAGTCAATGGGAAATTTTATGGAACAAGTAGAA	360
Qy	361	GAAGTCAATTAATCAAAAAATACAGATATGCAAGCAATTAAGCGCTTTCGGGAATTAGAA	420
Db	361	GAAGTCAATTAATCAAAAAATAGCAGATATGCAAGCAATTAAGCGCTTTCGGGAATTAGAA	420
Qy	421	GGATTAGGTAAATTAATCAAAATTAATCTAACTCGGCTTGAGAGATGGGAGAGAAATTCGA	480
Db	421	GGGCTTAGGGAAATTAATACCAATTAATCTAACTCGGCTTGAGAGATGGGAGAGAAATTCGA	480
Qy	481	AAATGGTTCAAGAGCCTTACGAGATGTCGGAATTCGAATTCGAATTCCTGGATAGTTTATTT	540
Db	481	AAATGGTTCAAGAGCCTTACGAGATGTTTCGAATTCGAATTCGAATTCCTGGATAGTTTATTT	540
Qy	541	ACGCAATATATGCACTCTTTTAGAGTGACAAATTTTGAAGTACCAATTCCTTACTGTATAT	600
Db	541	ACCCAAATATATGCACTCTTTTCGAGTGACAAATTTTGAAGTACCAATTCCTTACTGTATAT	600
Qy	601	GCAATGGGAGCCCAACTTCATTTACTGTTATTAAGGACGGTCAATTTTGGAGAGAGAA	660
Db	601	ACAAATGGCAGCAACCTACATTTACTTTTATTAAGGAGCGCATCAATTTTGGAGAGAGAA	660
Qy	661	TGGGGATGTCGAACAACTACTATTAATAAATTAATGATTCGTCAAAATGAAAATCTACTGCA	720
Db	661	TGGGGATTTGTCACAAAGCACTATTAATAACTACTATAATGTCAAATGAAAATCTACTGCA	720
Qy	721	GAATATTCTGATCACTGTGTAAAGTGGTATGAAACTGGTTTAGCAAAATTAAGGACGACG	780
Db	721	GAATATTCTGACCACTGTGTAAAGTGTATGAAACTGGTTTACGAAATTAAGGACGCTG	780
Qy	781	AGCGCTAAACAAATGGGTTGACTATTAACCAATTCCTTAGAGAAATGACATCGGCGGTTTTA	840
Db	781	AGCGCTAAACAAATGGGAATGACTATTAACCAATTCCTTAGAGAAATGACATTCGCGGTTTTA	840
Qy	841	GATGTTGTGCTATTAATTCCTAAATTAATGACACACGATCCCAATGGAAACGAAAGCA	900
Db	841	GAAGTTGTGCTATTAATTCCTAAATTAATGATAGCGTATGATCACTGCAACCAACAGCT	900

Qy	1981	CGGTAGATGAGACATATGAAAGCGGAACAAGATTTT	AGACGCGGAAAGCGAGTGAAT	2041
Db	1966	CGAGTAGATGAGACATATGAAAGCGGAACAAGATTTT	AGACGCGGAAAGCGAGTGAAT	2025
Qy	2041	GCCTTGTTTACGAATACAAAAGATGCGCTTATCGA	CCAGCGCGTAAACGATTTATGAGTGAAT	2100
Db	2026	GCCTTGTTTACGAATACAAAAGATGCGCTTATCGA	CCAGCGCGTAAACGATTTATGAGTGAAT	2085
Qy	2101	CAAGCGCAAACTTAGTGGAAATGCGCTTATCGAATG	ATGATTCCTCAAAATGGAACGATTCG	2160
Db	2086	CAAGCGCAAACTTAGTGGAAATGCGCTTATCGAATG	ATGATTCCTCAAAATGGAACGATTCG	2145
Qy	2161	TTATTTGATGCAAGTACGAGAGCGCAAAAGCGCTT	ACAGTACGAGTTCCTTTCCTTCAGAT	2220
Db	2146	TTATTTGATGCAAGTACGAGAGCGCAAAAGCGCTT	ACAGTACGAGTTCCTTTCCTTCAGAT	2205
Qy	2221	CCAGATTTTCCAAAGAGATAAATGCGAGAAAATGCT	GTGGACCGCAAGTACCGGAATTTGAGGTT	2280
Db	2206	CCAGATTTTCCAAAGAGATAAATGCGAGAAAATGCT	GTGGACCGCAAGTACCGGAATTTGAGGTT	2265
Qy	2281	ATAGAAAGCGCATGCTTTTATTTCAAAGCGCGCTT	ATCTACGCTTACAGGTCGAGAGAAATA	2340
Db	2266	ATAGAAAGCGCATGCTTTTATTTCAAAGCGCGCTT	ATCTACGCTTACAGGTCGAGAGAAATA	2325
Qy	2341	GATACCGGAACCTATCCAGCTATCTGTATCCAAAAG	TCTAGAGAGGTTGATTTAAACCA	2400
Db	2326	GATACCGGAACCTATCCAGCTATCTGTATCCAAAAG	TCTAGAGAGGTTGATTTAAACCA	2385
Qy	2401	TACACAGATATAGATTTGAGAGCGGTTTGTCCGAAG	CAAGTCAAGGATTTGGAATTTTCAACA	2460
Db	2386	TACACAGGTTATAGACTGTAGAGGATTTTGTGGAG	GATTTGCAAGGATTTATACG	2445
Qy	2461	ATTCTGTCATCAAAACGACCGGATTTGTAAAGATCT	ATCGGAGTCTATGCTGCGAGTGTA	2520
Db	2446	ATTCTGTCATCAAAACGACCGGATTTGTAAAGATCT	ATCGGAGTCTATGCTGCGAGTGTA	2505
Qy	2521	TCTCTCTGTTAACTCGGATGTTAGTATCAATTCGAT	CGAGCGAACAAAGTATGTGAATAGC	2580
Db	2506	CCTCTCTGTTAAACATATGATGTTAGATCAATTCG	ATCGGAGTCTATGCTGCGAGTGTA	2565
Qy	2581	CGTTTAGAAGTAGAAAAACCGTTCTCGTGTGAAG	CGCATGAGTTCTCTATTCCTATTTGATACA	2640
Db	2566	CGTTTAGAAGTAGAAAAACCGTTCTCGTGTGAAG	CGCATGAGTTCTCTATTCCTATTTGATACA	2625
Qy	2641	GGTGAATAATGATATCAATGAAAAATGCAAGATAT	GGGTTGCGATTTTAAAGATTAACGACCA	2700
Db	2626	GGAGAGCTGGATATCAATGAAAAATGCGGATATG	GGGTTGCGATTTTAAAGATTAACGACCA	2685
Qy	2701	GAGGATATGCAACCTCGGAAACCTAGATTTGCTG	ATGAGAGGACCTTTATTCAGAGGAC	2760
Db	2686	GAGGATATGCAACCTCGGAAACCTAGATTTGCTG	ATGAGAGGACCTTTATTCAGAGGAC	2745
Qy	2761	GCATTTAGAAGCTTTGCAAGAGAGAGAACAAACAG	TGGAAGTTCCAAATGCAAGAGAGACGT	2820
Db	2746	GCATTTAGAAGCTTTGCAAGAGAGAGAACAAACAG	TGGAAGTTCCAAATGCAAGAGAGACGT	2805
Qy	2821	GAAGAAACAGATAGAGTATATGCGATCGAAACAG	GCGGTAGTCTGTTATTTGCGCAT	2880
Db	2806	GAAGAAACAGATAGAGTATATGCGATCGAAACAG	GCGGTAGTCTGTTATTTGCGCAT	2865
Qy	2881	TATTCAGGATCAGCAACTGGAATCTCTGATGTAAG	ATTCAGATTTACTGCTGCGGCCCAAGAT	2940
Db	2866	TATTCAGGATCAGCAACTGGAATCTCTGATGTAAG	ATTCAGATTTACTGCTGCGGCCCAAGAT	2925
Qy	2941	CTGATACAGTCCATTCCTTACGTTATATCAAGAA	TGTTTCCAGAAATACCGAGATGAAC	3000
Db	2926	CTAATACAGTCCATTCCTTACGTTATATCAAGAA	TGTTTCCAGAAATACCGAGATGAAC	2985
Qy	3001	TATTCAGAGATTACAGATTTATCAGATTCGACTCC	ACACAGCGTGAGTTGTATGATCAG	3060
Db	2986	TATTCAGAGATTACAGATTTATCAGATTCGACTCC	ACACAGCGTGAGTTGTATGATCAG	3045
Qy	3061	CGAAATGCCATACCAAATGGTGTATTTTCGAAAT	TGGGTTAATGGAATGCAACGCTCT	3120

[illegible]

## RESULT 2

US-07-876-280-29  
: Sequence 29, Application US/07876280  
: Patent No. 5262158  
: GENERAL INFORMATION:  
: APPLICANT: Payte, Jewel M.  
: APPLICANT: Cannon, Raymond J.C.  
: APPLICANT: Bagley, Angela L.  
: TITLE OF INVENTION: No. 5262158el Bacillus thuringiensis  
: TITLE OF INVENTION: Controlling Acarides  
: NUMBER OF SEQUENCES: 30  
: CORRESPONDENCE ADDRESS:  
: ADDRESSEE: David R. Saliwanchik  
: STREET: 2421 N.W. 41st Street, Suite A-1  
: CITY: Gainesville  
: STATE: FL  
: COUNTRY: USA  
: ZIP: 32606  
: COMPUTER READABLE FORM:  
: MEDIUM TYPE: Floppy disk  
: COMPUTER: IBM PC compatible  
: OPERATING SYSTEM: PC-DOS/MS-DOS  
: SOFTWARE: Patent Release #1.0, Version #1.25  
: CURRENT APPLICATION DATA:  
: APPLICATION NUMBER: US/07/876,280  
: FILING DATE: 19920430  
: CLASSIFICATION: 514  
: ATTORNEY/AGENT INFORMATION:  
: NAME: Saliwanchik, David R.  
: REGISTRATION NUMBER: 31,794  
: REFERENCE/DOCKET NUMBER: M/S 104  
: TELECOMMUNICATION INFORMATION:  
: TELEPHONE: 904-375-8100  
: TELEFAX: 904-372-5800  
: INFORMATION FOR SEQ ID NO: 29:  
: SEQUENCE CHARACTERISTICS:  
: LENGTH: 3471 base pairs  
: TYPE: NUCLEIC ACID  
: STRANDEDNESS: double  
: TOPOLOGY: linear  
: MOLECULE TYPE: DNA (genomic)  
: HYPOTHEetical: NO  
: ANTI-SENSE: NO  
: ORIGINAL SOURCE:  
: ORGANISM: Bacillus thuringiensis  
: STRAIN: kumamotoensis  
: INDIVIDUAL ISOLATE: PS50C  
: IMMEDIATE SOURCE:  
: CLONE: E. coli NM522(pMYC2320) NRRL B-18769

GenCore version 5.1.3  
Copyright (c) 1993 - 2003 Compugen Ltd.

OM protein - protein search, using sw model

Run on: January 7, 2003, 05:12:43 ; Search time 26 Seconds  
(without alignments)  
1364.771 Million cell updates/sec

Title: US-10-032-717-2  
Perfect score: 6332  
Sequence: 1 MSPNNQNYEIIDATPSTV.....MSEETGTYIESVELIVDVE 1206

Scoring table: BLOSUM62

Gapop 10.0 , Gapext 0.5

Searched: 262574 seqs, 29422922 residues

Total number of hits satisfying chosen parameters: 262574

Minimum DB seq length: 0

Maximum DB seq length: 2000000000

Post-processing: Minimum Match 0  
Maximum Match 100  
Listing first 45 summaries

Database :

- Issued Patents AA:\*  
1: /cgn2\_6/ptodata/1/iaa/5A COMB pep.\*  
2: /cgn2\_6/ptodata/1/iaa/5B COMB pep.\*  
3: /cgn2\_6/ptodata/1/iaa/6A COMB pep.\*  
4: /cgn2\_6/ptodata/1/iaa/6B COMB pep.\*  
5: /cgn2\_6/ptodata/1/iaa/6CTUS COMB pep.\*  
6: /cgn2\_6/ptodata/1/iaa/backfiles1.pcp.\*

Pred. No. is the number of results predicted by chance to have a score greater than or equal to the score of the result being printed, and is derived by analysis of the total score distribution.

# SUMMARIES

Result No.	Score	Match	Length	DB ID	Description
1	4959.5	78.3	1169	1	US-08-315-468-4
2	3950.5	62.4	1157	1	US-07-876-280-30
3	3950.5	62.4	1157	1	US-07-812-180A-2
4	3950.5	62.4	1157	1	US-08-315-468-2
5	3950.5	62.4	1157	4	US-07-941-650A-2
6	3021	47.7	1149	1	US-07-915-203-2
7	3021	47.7	1149	1	US-08-272-887-2
8	3021	47.7	1149	2	US-08-789-449-2
9	2595.5	41.0	1157	2	US-08-532-547-5
10	2595.5	41.0	1157	2	US-08-379-656B-5
11	2595.5	41.0	1157	3	US-08-455-838-5
12	2595.5	41.0	1157	4	US-09-019-809-5
13	2595.5	41.0	1157	4	US-09-471-177-5
14	2590	40.9	1156	4	US-09-002-285-72
15	2558.5	40.4	1169	1	US-08-542-921-2
16	2558.5	40.4	1169	2	US-08-880-685-2
17	2558.5	40.4	1169	2	US-08-880-684-2
18	2449	38.7	1150	4	US-09-002-285-74
19	2419.5	38.2	1134	4	US-09-002-285-76
20	2397	37.9	1138	1	US-07-973-320-2
21	2391.5	37.8	1229	1	US-08-100-709-4
22	2391.5	37.8	1229	1	US-08-176-865-4
23	2391.5	37.8	1229	1	US-08-474-038-4
24	2391.5	37.8	1229	2	US-08-779-046-4
25	2391.5	37.8	1229	2	US-08-881-140-4
26	2380	37.6	1138	1	US-07-973-320-4
27	2353.5	37.2	1227	1	US-08-448-170-8

28 2353.5 37.2 1227 4 US-08-961-803-9 Sequence 9, Appli  
29 2335 36.9 1186 4 US-09-178-252-23 Sequence 23, Appli  
30 2333.5 36.9 1227 3 US-09-053-549-2 Sequence 2, Appli  
31 2312 36.5 1207 1 US-07-951-715A-7 Sequence 7, Appli  
32 2312 36.5 1207 2 US-08-459-448A-7 Sequence 7, Appli  
33 2312 36.5 1207 3 US-08-459-594B-7 Sequence 7, Appli  
34 2312 36.5 1207 3 US-08-459-594B-7 Sequence 7, Appli  
35 2312 36.5 1207 3 US-08-459-594B-7 Sequence 7, Appli  
36 2312 36.5 1207 3 US-09-053-549-8 Sequence 8, Appli  
37 2312 36.5 1207 4 US-09-547-422-7 Sequence 7, Appli  
38 2186.5 34.5 1189 1 US-08-602-737-2 Sequence 2, Appli  
39 2186.5 34.5 1189 4 US-09-001-982-2 Sequence 2, Appli  
40 2185.5 34.5 1189 6 518960-6 Patent No. 518960  
41 2184.5 34.5 1189 2 US-08-980-071-6 Sequence 6, Appli  
42 2184.5 34.5 1189 3 US-08-757-536-6 Sequence 6, Appli  
43 2184.5 34.5 1189 3 US-09-314-093-6 Sequence 6, Appli  
44 2184.5 34.5 1189 4 US-09-250-848-6 Sequence 6, Appli  
45 2184.5 34.5 1189 4 US-09-251-885-6 Sequence 6, Appli

## ALIGNMENTS

RESULT 1  
US-08-315-468-4  
; Sequence 4, Application US/08315468  
; Patent No. 5554534  
; GENERAL INFORMATION:  
; APPLICANT: Michaels, Tracy Ellis  
; APPLICANT: Focerrada, Luis  
; APPLICANT: Narva, Kenneth E.  
; TITLE OF INVENTION: Process for Controlling Scarab Pests  
; TITLE OF INVENTION: with Bacillus thuringiensis Isolates  
; NUMBER OF SEQUENCES: 6  
; CORRESPONDENCE ADDRESS:  
; ADDRESSEE: David R. Salivanchik  
; STREET: 2421 N.W. 41st Street, Suite A-1  
; CITY: Gainesville  
; STATE: FL  
; COUNTRY: USA  
; ZIP: 32606  
; COMPUTER READABLE FORM:  
; MEDIUM TYPE: Floppy disk  
; COMPUTER: IBM PC compatible  
; OPERATING SYSTEM: PC-DOS/MS-DOS  
; SOFTWARE: PatentIn Release #1.0, Version #1.25  
; CURRENT APPLICATION NUMBER: US/08/315,468  
; FILING DATE:  
; CLASSIFICATION: 424  
; PRIOR APPLICATION DATA:  
; APPLICATION NUMBER: US/08/014,941  
; FILING DATE: 01 FEB 1993  
; APPLICATION NUMBER: 07/828,430  
; FILING DATE: 30-JAN-1992  
; PRIOR APPLICATION DATA:  
; APPLICATION NUMBER: 07/808,316  
; FILING DATE: 16-DEC-1991  
; ATTORNEY/AGENT INFORMATION:  
; NAME: Salivanchik, David R.  
; REGISTRATION NUMBER: 31,794  
; REFERENCE/DOCKET NUMBER: MA73.C2  
; TELECOMMUNICATION INFORMATION:  
; TELEPHONE: 904-375-8100  
; TELEFAX: 904-372-5800  
; INFORMATION FOR SEQ ID NO: 4:  
; SEQUENCE CHARACTERISTICS:  
; LENGTH: 1169 amino acids  
; TYPE: amino acid  
; STRANDEDNESS: single  
; TOPOLOGY: linear  
; MOLECULE TYPE: protein  
; HYPOTHETICAL: YES

Instant Seq ID NO: 1  
vs protein encoded  
by Seq ID NO: 3  
of  
Michaels et al



; ANTI-SENSE: NO  
; ORIGINAL SOURCE:  
; ORGANISM: Bacillus thuringiensis  
; STRAIN: kumamotoensis  
; INDIVIDUAL ISOLATE: 50C  
; IMMEDIATE SOURCE:  
; LIBRARY: LambdaGEM-11 library of L. Focerrada  
; CLONE: 50C(b)  
; US-08-315-468-4

Query Match 78.3%; Score 4959.5; DB 1; Length 1169;  
Best Local Similarity 79.8%; Pred. No. 0;  
Matches 967; Conservative 68; Mismatches 128; Indels 49; Gaps 8;  
  
Qy 1 MSPNNQNEYIIDATPSTVSNDNRYPANEPNANLQNDYKYLKAGNASHYSGSP 60  
Db 1 MSPNNQNEYIIDATPSTVSNDNRYPANEPNANLQNDYKYLKAGNASHYSGSP 60  
  
Qy 61 EYLVSGDAAKAAIDIVGKLLGLGVPVPGPIVSLYTLQIDILWPSGKSKQWEIPMEQVE 120  
Db 61 EYVLSQDAAKAAIDIVGKLLGLGVPVPGPIVSLYTLQIDILWPSGKSKQWEIPMEQVE 120  
  
Qy 121 ELINOKIAEYARNKALSELGLGNNYQLVLTALPEWENPNSGRALRDVNRFEILDSLP 180  
Db 121 ELINOKIAEYARNKALSELGLGNNYQLVLTALPEWENPNSGRALRDVNRFEILDSLP 180  
  
Qy 181 TOYMPSPVNTNFEVPLTYVYMAANLHLLLLKQASIFGEWGWSTTYNNYDROMKLT 240  
Db 181 TOYMPSPVNTNFEVPLTYVYMAANLHLLLLKQASIFGEWGWSTTYNNYDROMKLT 240  
  
Qy 241 EYSDHCVMYETGLAKLGTSKQWVDVNOFREMTLAVLDVVALPNTDTTYPMETKA 300  
Db 241 EYSDHCVMYETGLAKLGTSKQWVDVNOFREMTLAVLDVVALPNTDTTYPMETKA 300  
  
Qy 301 QUTREVTYDPLGAVNVSIGSWTDKAPSGVIESVTRPHVDYITGLTYTQSGRIS 360  
Db 301 QUTREVTYDPLGAVNVSIGSWTDKAPSGVIESVTRPHVDYITGLTYTQSGRIS 360  
  
Qy 361 ARYIRHAGHOISYHRSRGNLQMYGTQNKHLSTSTFTFYNDIYKTLSDAVLLDV 420  
Db 361 DRYRYMAGHOISYHRSRGNLQMYGTQNKHLSTSTFTFYNDIYKTLSDAVLLDV 420  
  
Qy 421 YEGTYIFPGNPEVEPMNQLNNTKTLKYNPVSKDILASTROSELEPPETSDNYE 480  
Db 421 YEGTYIFPGNPEVEPMNQLNNTKTLKYNPVSKDILASTROSELEPPETSDNYE 480  
  
Qy 481 SYSHRLCHITIPATGNTTGLVPVFSWTHRSADLNTIYSDKITQIPAVKCHNDLPFPV 540  
Db 481 SYSHRLCHITIPATGNTTGLVPVFSWTHRSADLNTIYSDKITQIPAVKCHNDLPFPV 540  
  
Qy 541 VKPGCHTGGDLLOVNRSTGSGVTLFLARYGLALEKAGYRRLRYATDADIVLHV 596  
Db 540 IKRGYTGGLVALTDIGS-----CEPOMIPPEQSPRIRRYASNETSVISLYGLNQ 593  
  
Qy 597 DAQIQMPT-MWGE-DLSTKTFKADATITLNLATSSALKNLKNLGEDPNTLSGIVYV 654  
Db 594 SGLTFNGTYSNGKNDLTNDPKYIEYPRVSVNASSNQ-RLSIGIQTNTNL----FIL 649  
  
Qy 655 DRIFIPVDYETAEODLEAKKANVALFTNTKGLRPGVTDYEVNQAANLVECLSDLY 714  
Db 650 DRIFIPVDYETAEODLEAKKANVALFTNTKGLRPGVTDYEVNQAANLVECLSDLY 709  
  
Qy 715 PNEKELLEDAVREAKLSEARNLQDPDPOZINGENGHTASTGIEVEDALFKRYRL 774  
Db 710 PNEKELLEDAVREAKLSEARNLQDPDPOZINGENGHTASTGIEVEDAVPKRYRL 769  
  
Qy 775 PGAREIDTETTYLYQKVEEGLKPYTRYLRGFPVSSQGLSIFTRHQTNRIVKNVPD 834  
Db 770 PGAREIDTETTYLYQKVEEGLKPYTRYLRGFPVSSQGLSIFTRHQTNRIVKNVPD 829  
  
Qy 835 DLLPDSVSNDSGKINRCSOKYNSRLVENRSGAHEFSIPDGTGVDYNNAGIHWG 894  
Db 830 DLLPDSVSNDSGKINRCSOKYNSRLVENRSGAHEFSIPDGTGVDYNNAGIHWG 889

Qy 895 FKITDPGATVATLGNLLEVEGPLSGDLERLQREZOOVKIOMTRERRETDERRYNASKQAV 954  
Db 890 FKITDPGATVATLGNLLEVEGPLSGDLERLQREZOOVKIOMTRERRETDERRYNASKQAV 949  
  
Qy 955 DRLVADYQDQOLNPDVEITDLTAQDLIQSIPIVYNNEMPEIPGANYTKFTLTDLRLOQA 1014  
Db 950 DRLVADYQDQOLNPDVEITDLTAQDLIQSIPIVYNNEMPEIPGANYTKFTLTDLRLOQA 1009  
  
Qy 1015 WSLYDQBNALPNDGFRANGLSNMNNATPGVEVQOQINHTSVLVINENDEQVSOQFTVQPNQRY 1074  
Db 1010 WSLYDQBNALPNDGFRANGLSNMNNATPGVEVQOQINHTSVLVINENDEQVSOQFTVQPNQRY 1069  
  
Qy 1075 VLRYTARKEGVGVYSIRDOGNOTETLTFSASDYDNGMYTQVSKNTNGTNNAYNQ 1134  
Db 1070 VLRYTARKEGVGVYSIRDOGNOTETLTFSASDYDNGMYTQVSKNTNGTNNAYNQ 1108  
  
Qy 1135 ASBTNGVNNMNTAGNTNGTNTSVNDQGYITKTVPITPYDQMIENSETGT 1194  
Db 1109 -----MYDTQASNTNGTNTSVNIMKPAISRTKTDVSISSVYNNQMIETSETGT 1157  
  
Qy 1195 YIESVELIVDVE 1206  
Db 1158 YIESVELIVDVE 1169

## RESULT 2

US-07-876-280-30  
; Sequence 30, Application US/07876280  
; Patent No. 5262158  
; GENERAL INFORMATION:  
; APPLICANT: Payne, Jewel M.  
; APPLICANT: Calton, Raymond J.C.  
; APPLICANT: Bagley, Angela L.  
; TITLE OF INVENTION: No. 5262158el Bacillus thuringiensis Isolates for  
; TITLE OF INVENTION: Controlling Acarides  
; NUMBER OF SEQUENCES: 30  
; CORRESPONDENCE ADDRESS:  
; ADDRESSEE: David R. Saliwanchik  
; STREET: 2421 N.W. 41st Street, Suite A-1  
; CITY: Gainesville  
; STATE: FL  
; COUNTRY: USA  
; ZIP: 32606  
; COMPUTER READABLE FORM:  
; MEDIUM TYPE: Floppy disk  
; COMPUTER: IBM PC compatible  
; OPERATING SYSTEM: PC-DOS/MS-DOS  
; SOFTWARE: Patent In Release #1.0, Version #1.25  
; CURRENT APPLICATION DATA:  
; APPLICATION NUMBER: US/07/876,280  
; FILING DATE: 19920430  
; CLASSIFICATION: 514  
; ATTORNEY/AGENT INFORMATION:  
; NAME: Saliwanchik, David R.  
; REGISTRATION NUMBER: 31,794  
; REFERENCE/DOCKET NUMBER: M/S 104  
; TELECOMMUNICATION INFORMATION:  
; TELEPHONE: 904-375-8100  
; TELEFAX: 904-372-5800  
; INFORMATION FOR SEQ ID NO: 30:  
; SEQUENCE CHARACTERISTICS:  
; LENGTH: 1157 amino acids  
; TYPE: AMINO ACID  
; STRANDEDNESS: single  
; TOPOLOGY: linear  
; MOLECULE TYPE: protein  
; HYPOTHETICAL: YES  
; ANTI-SENSE: NO  
; ORIGINAL SOURCE:  
; ORGANISM: Bacillus thuringiensis  
; STRAIN: kumamotoensis  
; INDIVIDUAL ISOLATE: PS50C

# Appendix B

# Cloning and study of the expression of a novel *cryIIa*-type gene from *Bacillus thuringiensis* subsp. *kurstaki*

S. Tounsi, N. Zouari and S. Jaoua

Laboratoire des Biopesticides, Centre de Biotechnologie de Sfax, Sfax, Tunisia

2002/239; received 6 June 2002, revised 13 February 2003 and accepted 21 February 2003

## ABSTRACT

S. TOUNSI, N. ZOUARI AND S. JAOUA. 2003.

**Aims:** Cloning and expression of a new *cryIIa*-type gene of *Bacillus thuringiensis*.

**Methods and Results:** PCR amplification, using gene *cryII*-specific primers revealed the presence of such a gene in the strain BNS3 of *Bacillus thuringiensis* subsp. *kurstaki*. The cloning and sequencing from BNS3 of the *cryIIa*-type gene, called *crybns3-3*, showed an open reading frame of 2160-bp, encoding a protein of 719 amino acid residues. Both nucleotide and amino acid sequences similarity analysis revealed that the *crybns3-3* is a new *cryIIa*-type gene, presenting several differences from the *cryIIa*-type genes. The study of the expression of *crybns3-3* by Northern blot and RT-PCR showed that it was transcribed. The expression of *crybns3-3* under the control of BtI and BtII promoters revealed that *Crybns3-3* would co-crystallize with the endogenous delta-endotoxins.

**Conclusions:** *crybns3-3* is a novel *cryIIa* gene isolated from *B. thuringiensis* subsp. *kurstaki* strain BNS3.

**Significance and Impact of the Study:** The characteristics of *crybns3-3* indicate that it is a new *cryIIa*-type gene. Amino acid residue substitutions presented in *Crybns3-3* could be exploited for both toxicity and specificity studies. *Crybns3-3* would interact and co-crystallize at least partially with the endogenous delta-endotoxins of BNS3, and then participate in the formation of the parasporal crystal inclusions.

**Keywords:** *Bacillus thuringiensis*, cloning, *cryIIa*, DNA sequence, expression.

## INTRODUCTION

*Bacillus thuringiensis* (Bt) is a Gram-positive bacterium characterized by the production of insecticidal crystal proteins during sporulation, exhibiting a wide variety of insecticidal specificities towards lepidopteran, coleopteran and dipteran insect species (Höfte and Whiteley 1989). These proteins are termed delta-endotoxins because of their intracellular location and have been used for many years as successful biological insecticides. Delta-endotoxins encoded by *cry* genes have been classified as Cry1-2-3... proteins, depending on their host specificity and their amino acid sequences (Höfte and Whiteley 1989). The occurrence of insect resistance to Cry proteins (Tabashnik *et al.* 1990) has been attributed to a reduction in the affinity of the proteolytically activated Cry toxin for binding to the

membrane of mid-gut epithelial cells of the larvae (Tabashnik *et al.* 1990). In order to prevent the continuous development of resistance to Bt insecticides, a search for novel Bt strains (Zouari *et al.* 2002a) harbouring new *cry* genes has been undertaken (Shin *et al.* 1995). Thus, Tailor *et al.* (1992) reported a new class of *cry* genes encoding proteins that could not be found in solubilized Bt crystal preparations. Such a gene is designated *cryIIa* (Crickmore *et al.* 1998) on the basis of the following criteria: (i) it has 62% sequence homology with *cryIBa* (Tailor *et al.* 1992) and (ii) when expressed in *Escherichia coli*, the encoded 81 kDa protein, showed specificity and insecticidal activity against lepidopterans (*Ostrinia nubilalis*) and coleopterans (*Leptinotarsa decimlineata*) (Tailor *et al.* 1992). However, other groups (Shin *et al.* 1995) have reported the cloning and characterization of silent *cryIIa*-type genes in different Bt strains. Recently, Masson *et al.* (1998), reported that the Bt subsp. *aizawai* strain HD133 expressed a *cryIIa*-type gene at the early stages of the sporulation phase.

Correspondence to: S. Jaoua, Laboratoire des Biopesticides, Centre de Biotechnologie de Sfax, PO Box K, 3038, Sfax, Tunisia (e-mail: samir.jaoua@cbs.rnrt.tn).

In this paper, we report the cloning, the nucleotide sequence and the study of the expression of a new *cryIIa*-type gene named *crybns3-3*, isolated from the strain BNS3 (Tounsi *et al.* 1999) of Bt, and whose promoter has been previously identified (Tounsi and Jaoua 2002).

## MATERIALS AND METHODS

### Bacterial strains, plasmids and growth conditions

The strain BNS3 employed in the present work is Bt *kurstaki* subsp. H3a, 3b, 3c (Tounsi *et al.* 1999). This strain produces bipyramidal parasporal crystals exhibiting insecticidal activities (Tounsi *et al.* 1999). This strain was selected for the large-scale production of bioinsecticides (Zouari *et al.* 1998, 2002b). The strain BNS3Cry<sup>-</sup> is an acrySTALLIFEROUS strain derivative of BNS3 (Tounsi *et al.* 1999). *Escherichia coli* DH5 $\alpha$  obtained from Amersham (Sunnyvale, CA, USA), was used as a cloning host. Plasmid pBluescript II KS(+) (Stratagene, La Jolla, CA, USA) was the vector used to construct the genomic library. Plasmid pHTBlue, a derivative of pHT3101, was made in the laboratory by substituting the multiple cloning site of the latter for that of the plasmid pBluescript II KS (unpublished data). It was used to study the expression of *cry* genes in Bt. Plasmid pHTBcrybns3-3 was constructed in this work by cloning the gene *crybns3-3* of BNS3 in the plasmid pHTBlue, downstream of the BtI and BtII promoters. Strains BNS3(pHTBcrybns3-3) and BNS3Cry<sup>-</sup>(pHTBcrybns3-3) were constructed in this work by transferring the plasmid pHTBcrybns3-3, respectively, to BNS3 and BNS3Cry<sup>-</sup>. LB broth (Sambrook *et al.* 1989) was used for growth of Bt and *E. coli*. Glucose medium was used for delta-endotoxin production by Bt strains (Zouari *et al.* 1998).

### Preparation of DNA and DNA probe, and PCR amplification

Plasmid DNA was extracted from Bt strains using the alkali lysis method including a lysozyme treatment step (Sambrook *et al.* 1989). Primers cry5A and cry5B (Shin *et al.* 1995) were synthesized by the Centre de Génétique Moléculaire, CNRS, GENSET, Orsay, France. PCR was accomplished as described by Jaoua *et al.* (1996). The *cryIIa*-specific probe was prepared from BNS3, by PCR using primers cry5A and cry5B.

### Nucleic acid hybridization

Restricted DNAs were size separated by electrophoresis in horizontal 0.8% agarose slab gels and transferred to nylon membrane (Hybond N<sup>+</sup>; Amersham) (Sambrook *et al.* 1989). Prehybridization and hybridization of filters were carried out as described in Sambrook *et al.* (1989).

### Cloning experiment, transformation procedures and DNA sequencing

Plasmid DNA purified from the BNS3 was digested with *Cla*I and *Bam*III and separated by electrophoresis through a 0.8% agarose gel. DNA fragments (3-kb) were ligated to pBluescript II KS digested with both *Cla*I and *Bam*III. Transformation of *E. coli* DH5 $\alpha$  was performed according to the manufacturer's protocol (Bethesda Research Laboratories, Bethesda, MD, USA). Transformation of BNS3Cry<sup>-</sup> and BNS3 was accomplished by electroporation using a Bio-Rad Gene Pulser (BioRad, Hercules, CA, USA), according to Lereclus *et al.* (1989).

DNA sequences were obtained according to the dideoxy-chain termination method using the Thermo Sequenase cycle sequencing kit (Amersham). Sequence comparisons were performed using Blast through the NCBI E-mail server.

The DNA sequence of *crybns3-3* had been deposited in the EMBL and Gene Bank data bases under the accession number AJ315121.

### RNA isolation, RT-PCR, Northern blot analysis

Total RNA from BNS3 and BNS3Cry<sup>-</sup> was isolated from cells grown in 10 ml of LB medium at 30°C for 15 h (Tounsi and Jaoua 2002). Bacterial cells were centrifuged at 5000 g for 5 min at 4°C. Pellets were suspended in 2 ml of buffer (25% w/v, sucrose; 50 mmol l<sup>-1</sup> Tris-HCl (pH 8.0); 0.25 mmol l<sup>-1</sup> EDTA) containing 1 mg ml<sup>-1</sup> lysosyme, and incubated for 15 min at 37°C. The obtained protoplasts were harvested by centrifugation and suspended in 0.6 ml lysis buffer (3 mmol l<sup>-1</sup> EDTA; 200 mmol l<sup>-1</sup> NaCl; 0.5% SDS) and 0.6 ml of phenol and centrifuged at 12 000 g for 5 min at 4°C. The aqueous phase containing total RNA was treated twice with phenol/chloroform/isoamylalcohol (25 : 24 : 1, by vol.). After precipitation with 100% ethanol, RNA pellets were washed with 1 ml 70% (v/v) ethanol, dried and dissolved in 50  $\mu$ l of sterilized bi-distilled water treated with diethyl pyrocarbonate. RT-PCR experiments were accomplished by using ready-to-go RT-PCR kit (Amersham) and purified RNA was treated with DNase I (RNase-free, Amersham). For Northern blot analysis, 20  $\mu$ g of total RNA was electrophoresed in 1% agarose gel containing 1.2 mol l<sup>-1</sup> formaldehyde. The RNA was transferred to nylon membrane (Hybond N<sup>+</sup>; Amersham) using 20 $\times$  SSC (1 $\times$  SSC is 0.15 mol l<sup>-1</sup> NaCl, 0.015 mol l<sup>-1</sup> sodium citrate).

### Delta-endotoxin determination

Delta-endotoxin was determined according to Zouari *et al.* (1998).

ATGAAACTAAAGAATCAAGATAAGCATCAAAGTTTTTCTAGCAATGCGAAAGTAGATAAAATCTCTACGGATTCACTAAAAAATGAAACAGATATAGAAT 100  
 M K L K N Q D K H Q S F S S N A K V D K I S T D S L K N E T D I E  
 TACAAAACATTAAATCATGAGATTGTTTGAAATGTCTGAGTATGAAATGTAGAGCCGTTTGTAGTGCATCAACAATTCAAACAGGTATTGGTATTGC 200  
 L Q N I N H E D C L K M S E Y E N V E P F V S A S T I Q T G I G I A  
 GGGTAAAATACCTGGTACCCTAGGCGTTCTTTTGCAAGACAAGTAGCTAGTCTTTATAGTTTTATCTTAGGTAGCTATGGCCTAAGGGGAAAAATCAA 300  
 G K I L G T L G V P F A G Q V A S L Y S F I L G E L W P K G K N Q  
 TGGGAAATCTTTATGGAACATGTAGAAGAGATTATTAATCAAAAAATATCAACTTATGCAAGAAATAAGCACTTACAGACTTGAAAGGATTAGGAGATG 400  
 W E I F M E H V E E I I N Q K I S T Y A R N K A L T D L K G L G D  
 CCTTAGCTGTCTACCATGATTTCGCTTGAAAGTTGGGTTGGAATCGTAATAACAAGGGCTAGGAGTGTGTCAAGAGCCAATATATCGCATTAGAATT 500  
 A L A V Y H D S L E S W V G N R N N T R A R S V V K S Q Y I A L E L  
 GATGTTGCTTCAGAACTACCTTCTTTTGCAAGTGTCTGGAGAGGAGGTACCATTATTACCGATATATGCCCAAGCTGCAAAATTTACATTGTGTCTATTA 600  
 M F V Q K L P S F A V S G E E V P L L P I Y A Q A A N L H L L L L  
 AGAGATGCATCTATTTTTGGAAGAGTGGGGATTATCATCTTCAGAAATTTCAACATTTTATAACCGTCAAGTCAAGCAGCAGGAGATTATCCGACC 700  
 R D A S I F G K E W G L S S S E I S T F Y N R Q V E R A G D Y S D  
 ATGTGTGAAATGGTATAGCAGAGGTCTAAATAAATTGAGGGGTACAAATGCCGAAAGTTGGGTACGATATAATCAATTCGTAGAGACATGACTTTAAT 800  
 H C V K W Y S T G L N N L R G T N A E S W V R Y N Q P R R D M T L M  
 GGTACTAGATTTAGTGGCACTATTTCCAAGCTATGATACACAAATGTATCCAATTAATACTACAGCCCACTTCAAGAGAAGTATATACAGACGCAATT 900  
 V L D L V A L F P S Y D T Q M Y P I K T T A Q L T R E V Y T D A I  
 GGGACAGTACATCCGCATCCAAGTTTACAAGTACGACTTGGTATAATAATAATGCACCTTCGTTCTCTGCCATAGAGGCTGCTGTTGTTGAAACCCGC 1000  
 G T V H P H P S F T S T T W Y N N N A P S F S A I E A A V V R N P  
 ATCTACTCGATTTTCTAGAACAAGTTACAATTTACAGCTTATTAAGTCGATGGAGTAACACTCAGTATATGAATATGTGGGGAGGACATAAATAGAAATT 1100  
 H L L D F L E Q V T I Y S L L S R W S N T Q Y M N M W G G H K L E F  
 CCGAACAATAGGAGGAACGTTAAATATCTCAACACAAGGATCTACTAATCTCTATTAATCCTGTAAACATTACCGTTCCTTCGAGACGCTCTATAGG 1200  
 R T I G G T L N I S T Q G S T N T S I N P V T L P F T S R D V Y R  
 ACTGAATCATTGGCAGGGCTCAATCTATTTTAACTCAACCTGTTAATGGAGTACCTAGGGTTGATTTCATTGGAAATTCGTCACACATCCGATCGCAT 1300  
 T E S L A G L N L F L T Q P V N G V P R V D F H W K P V T H P I A  
 CTGATAATTTCTATTATCCAGGGTATGCTGGAATTGGGACGCAATTACAGGATTGAGAAAATGAATTACCACTGGAAGCAACAGGACAGCCAAATTATGA 1400  
 S D N F Y Y P G Y A G I G T Q L Q D S E N E L P P E A T G Q P N Y E  
 ATCTTATAGTCATAGATTATCTCATATAGGACTCATTTACGATCAGATGTGAAAGCATTGGTATATTCTTGGACGCATCGTAGTCAGATCGTACAAAT 1500  
 S Y S H R L S H I G L I S A S H V K A L V Y S W T H R S A D R T N  
 ACAATTGAGCCAAATAGCATTACACAAATACCATTAGTAAAAGCTTTCAATCTGTCTTCAGGTGCCGCTGTAGTGAGAGGAGCAGGATTACAGGTGGGG 1600  
 T I E P N S I T Q I P L V K A P N L S S G A A V V R G P G F T G G  
 ATATCCTTCGAAGAACAATACTGGTACATTTGGGGATATACGAGTAAATATTAATCCACCATTTCACAAAGATATCGCGTGAGGATTTCGTATGCTTC 1700  
 D I L R R T N T G T F G D I R V N I N P P F A Q R Y R V R I R Y A S  
 TACCACAGATTTACAATTCATACGTCATTAACGGTAAAGCTATTAATCAAGGTAATTTTTCAGCAACTATGAATAGAGGAGGAGGACTTAGACTATAAA 1800  
 T T D L Q F H T S I N G K A I N Q G N F S A T M N R G E D L D Y K  
 ACCTTTAGAACTGTAGGCTTTACCACTCCATTTAGCTTTTATAGATGTACAAAGTACATTACAATAGGTGCTTGGAACTTCTCTTCAGGTAACGAAGTTT 1900  
 T P R T V G F T T P F S F L D V Q S T F T I G A W N F S S G N E V  
 ATATAGATAGAATTGAATTTGTTCCGGTAGAAGTAACATATGAGGCAGAATATGATTTTGAAAAAGCGCAAGAGAAGGTTACTGCCTGTTTACATCTAC 2000  
 Y I D R I E F V P V E V T Y E A E Y D F E K A Q E X V T A L F T S T  
 GAATCCAAGAGGATTAAAAACAGATGTAAAGGATTATCATATTGACCAGGTATCAAATTTAGTAGAGTCTCTATCAGATGAATTTCTATCTTGATGAAG 2100  
 N P R G L K T D V K D Y H I D Q V S N L V E S L S D E F Y L D E K  
 AGAGAATTATTCAGATAGTTAAATACGCGAAGCAACTCCATATTGAGCGTAACATGTAG 2150  
 R E L F E : V K Y A N E L H I E R N M

Fig. 1 Nucleotide sequence (EMBL accession number AJ315121) and deduced amino acid sequence of *arybns3-3*. The underlined ATG and TGA correspond, respectively, to the start and stop codons of *crybns3-3*. The predicted *Bacillus* secretion signal peptide has been italicized

## RESULTS

### Southern hybridization and cloning of a *cryIIa*-type gene from BNS3

The presence of a *cryIIa*-type gene in BNS3 was demonstrated by PCR amplification of an expected 0.7-kb fragment (Tounsi *et al.* 1999). Plasmid DNA of BNS3 digested with both *Cla*I and *Bam*HI restriction enzymes, was analysed by Southern blot hybridization with the 0.7-kb radio-labelled probe. A band corresponding to 3-kb *Cla*I-*Bam*HI restriction fragment was visualized on the autoradiogram (unpublished data). Thus, a library of the 3-kb *Cla*I-*Bam*HI fragments was constructed from BNS3 plasmid DNA and screened at strong stringency conditions using the same 0.7-kb probe mentioned above. The restriction enzyme analysis of the positive recombinant clones revealed the presence of a *cryIIa*-type gene in BNS3. The cloned gene was called *crybns3-3*.

### Sequence analysis of *crybns3-3*

Analysis of the nucleotide sequence (EMBL accession no. AJ315121) (Fig. 1) of the cloned 3-kb *Cla*I-*Bam*HI fragment showed the presence of an open reading frame of 2160 bp encoding a protein of 719 amino acid residues having a predicted molecular mass of 81 kDa. The search for sequence similarity of *crybns3-3* with the known genes, using the Blast program, revealed several differences with the published *cryIIa*-type genes (Fig. 2). When compared with *cryIIa1* (Tailor *et al.* 1992), there were substitutions at positions 697 of T for G (transversion), 2133 of G for C (transversion) and 2134 of C for G (transversion), resulting in the substitution of Tyr<sup>233</sup> for Asp (domain I), Lys<sup>711</sup> for Asn and Gln<sup>712</sup> for Glu (domain III). Compared with *cryIIa2* (Gleave *et al.* 1993), there were substitutions at positions 2133 of G for C and 2134 of C for G, resulting in the substitution of Lys<sup>711</sup> for Asn and Gln<sup>712</sup> for Glu (domain III). Compared with *cryIIa3* (Shin *et al.* 1995), there were substitutions at positions 697 of T for G, resulting in the substitution in the amino acids sequence of Tyr<sup>233</sup> for Asp (domain I). Compared with *cryIIa4* (Kostichka *et al.* 1996), there were substitutions at positions 1328 of T for C (transition), 2133 of G for C, 2134 of C for G and 1398 of C for T, resulting in the substitution in the amino acids sequence of, respectively, Val<sup>443</sup> for Ala (domain II), Lys<sup>711</sup> for Asn and Gln<sup>712</sup> for Glu (domain III).

### Study of the expression of the *crybns3-3* gene in the wild strain BNS3

Although DNA dot-blot analysis and/or PCR analysis confirmed the presence of a *cryIIa*-type gene in most Bt strains, CryIIa proteins were not detected in solubilized

Δ-Endotoxins	1	233	443	711	712	719
	↓	▼	▼	▼▼		↓
CryBNS3-3	: MKLKN...DYS <sup>233</sup> HCV...PGY <sup>443</sup> IGIG...YAN <sup>711</sup> ELHIERNM					
CryIIa1	: MKLKN...DYS <sup>233</sup> HCV...PGY <sup>443</sup> IGIG...YAK <sup>711</sup> OLHIERNM					
CryIIa2	: MKLKN...DYS <sup>233</sup> HCV...PGY <sup>443</sup> IGIG...YAK <sup>711</sup> OLHIERNM					
CryIIa3	: MKLKN...DYS <sup>233</sup> HCV...PGY <sup>443</sup> IGIG...YAN <sup>711</sup> ELHIERNM					
CryIIa4	: MKLKN...DYS <sup>233</sup> HCV...PGY <sup>443</sup> IGIG...YAK <sup>711</sup> OLHIERNM					

Fig. 2 Comparison of the amino acid sequence encoded by *crybns3-3* with those encoded by the other *cryIIa*-type genes. Only the regions containing differences are presented. The vertical downward arrows indicate amino acid position. The black boxes represent the residues showing variations

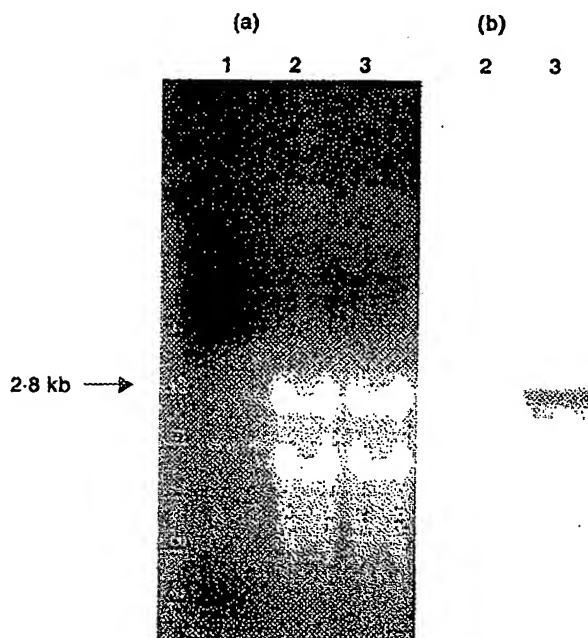
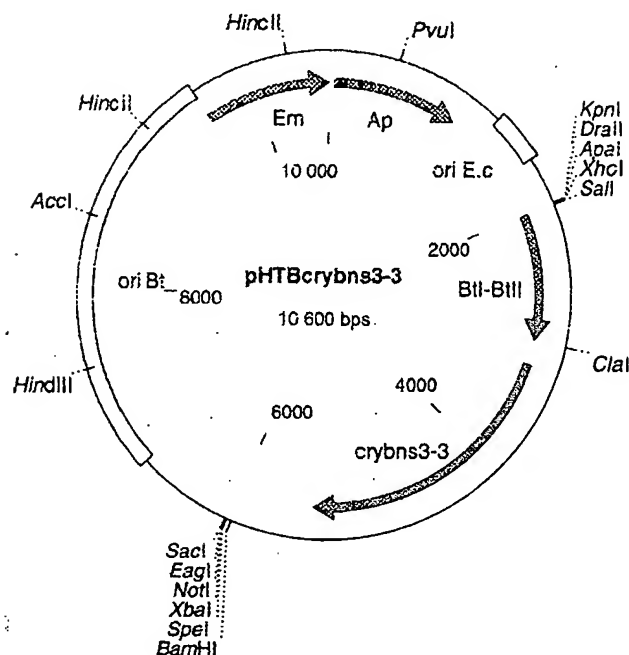


Fig. 3 Northern blot analysis of *crybns3-3* transcript (digitally processed). (a) Total RNA, extracted as described in material and methods from BNS3 and BNSCry<sup>-</sup>, and electrophoresed in 1% agarose gel containing 1.2 mmol l<sup>-1</sup> formaldehyde. Lanes: 1, RNA Molecular weight markers (Amersham): 9.46, 6.23, 3.91, 2.80, 1.90, 0.87, 0.56, 0.36-kb; 2, total RNA of BNS3Cry<sup>-</sup>; 3, total RNA of BNS3. (b) Northern blot of RNAs hybridized to a *cryIIa*-specific probe (lane designation are the same as for panel a)

crystal preparations (Tailor *et al.* 1992; Gleave *et al.* 1993). However, Kostichka *et al.* (1996) and Masson *et al.* (1998) reported, respectively, that Bt AB88 expressed a *cryIIa*-type gene in early stationary phase and Bt HD133 expressed a *cryIIa*-type gene at both the T2 and T5 stages of the sporulation phase. In our case, Crybns3-3 proteins were not found by SDS-PAGE of crystal preparation (unpublished



**Fig. 4** Restriction map of the plasmid pHTBcrybs3-3. The construction of the plasmid is described in the text. Open boxes contain the replication origins of, respectively, Bt (ori Bt) and *Escherichia coli* (ori E.c.). Filled arrows indicate the direction of transcription of the erythromycin resistance gene (Em), ampicillin resistance gene (Ap), *crybs3-3* gene and the orientation of the double promoters BtI and BtII.

**Table 1** Evidence of the Crybs3-3-mediated delta-endotoxin production improvement

Strain	CFU ( $10^7$ C ml $^{-1}$ )	Production of delta-endotoxin (mg l $^{-1}$ )	Improvement of delta-endotoxin production
BNS3(pHTBlue)	100	960	Control
BNS3(pHTBcrybs3-3)	100	1120	16.6 %

data). Analysis of total RNA of BNS3 by Northern blot demonstrated that BNS3 expressed a *cryIIa*-type gene with a transcript size of 2.8-kb (Fig. 3). RT-PCR profile showed the amplification of an expected 0.7-kb fragment (using cry5A and cry5B primers), which confirm the expression of *crybs3-3* (unpublished data).

#### Expression of *crybs3-3* under the control of the BtI and BtII promoters

In order to improve the expression of *crybs3-3* during the early and later stages of sporulation, the *crybs3-3* was cloned downstream of the double promoters BtI and BtII (Sedlak *et al.* 2000). First, the 3-kb *ClaI*-*BamHI* fragment containing the *crybs3-3* gene was cloned in between the *ClaI* and

*BamHI* sites of the plasmid pHTBlue. Then, a 1-kb *SmaI* fragment containing the BtI and BtII promoters obtained by PCR amplification-*SmaI* digestion (unpublished results) was cloned in the *HincII* site of the recombinant plasmid pHTBcrybs3-3. The resulting recombinant plasmid, containing the *crybs3-3* gene under the control of the BtI and BtII promoters designated pHTBcrybs3-3 (Fig. 4), was transferred by electroporation to both BNS3 and BNS3Cry $^-$ . Microscopic examination of sporulated recombinant transformants BNS3Cry $^-$  (pHTBcrybs3-3) revealed the lack of parasporal crystal inclusions. However, using polyclonal antibodies against *cryIA* genes, the Crybs3-3 was detected in the supernatant by ELISA. However, a comparison of delta-endotoxin production by the different strains (Table 1) showed that BNS3(pHTBcrybs3-3) produced 16% more delta-endotoxins than BNS3(pHTBlue) used as a control.

#### DISCUSSION

It is known that Cry proteins, belonging to the same type, are closely related. In the present work we have isolated a novel *cryIIa*-type gene, *crybs3-3*, encoding a protein of 719 amino acid residues, having a predicted molecular mass of 81 kDa. Hence, the Crybs3-3 protein sequence is different at several sites from those of the reported CryIIa proteins. As regards the effect of the reported substitutions in the *crybs3-3* gene, we could expect modifications in toxicity and specificity of the encoded delta-endotoxins. Deep investigations of such effects should be performed. Although delta-endotoxins encoded by *cryIIa1* and *cryIIa2* genes included single amino acid differences in domain I, they exhibited a different insecticidal activity spectrum, inferring different specificities (Gleave *et al.* 1993). Such results reinforce the idea that substitutions presented by *crybs3-3* could be of interest for studies on toxicity.

We demonstrated that BNS3 expressed a *cryIIa*-type gene with a transcript size of 2.8-kb. By ELISA, the Crybs3-3 protein was detected at the early stages of sporulation, in the culture media of BNS3. This indicates that Crybs3-3 delta-endotoxin is secreted probably due to the presence of a putative signal peptide in the N-terminal domain I (Kostichka *et al.* 1996).

In order to improve its expression, *crybs3-3* was cloned downstream both of the promoters BtI and BtII in the shuttle vector pHTBlue, and transferred to both BNS3 and BNS3Cry $^-$ . The lack of parasporal crystal inclusions in the sporulated recombinant transformants BNS3Cry $^-$  (pHTBcrybs3-3) demonstrates that even when transcribed during the early and later stages of sporulation, the delta-endotoxin Crybs3-3 does not crystallize. The enhancement of delta-endotoxin production by BNS3(pHTBcrybs3-3) clearly shows that Crybs3-3 does not crystallize when it is

alone, but can interact and co-crystallize partially with the endogenous delta-endotoxins of BNS3, and participate in the formation of parasporal crystal inclusions.

## ACKNOWLEDGEMENTS

We thank Mr Binesh Shrestha for correcting the English language. We also thank Mrs Najeh Belguith Hassine for her technical assistance. This work was supported by grants from the Tunisian Secrétariat d'état à la Recherche Scientifique et à la Technologie.

## REFERENCES

- Crickmore, N., Zeigler, D.R., Feitelson, J., Schnepf, H.E., Van Rie, J., Lereclus, D., Baum, J. and Dean, D.H. (1998) Revision of the nomenclature for the *Bacillus thuringiensis* pesticidal crystal proteins. *Microbiology and Molecular Biology Reviews* 62, 807–813.
- Gleave, A.P., Williams, R. and Hedges, J. (1993) Screening by polymerase chain reaction of *Bacillus thuringiensis* serotypes for the presence of *cryV*-like insecticidal protein genes and characterization of *cryV* gene cloned from *B. thuringiensis* subsp. *kurstaki*. *Applied and Environmental Microbiology* 59, 1683–1687.
- Höfte, H. and Whiteley, H.R. (1989) Insecticidal crystal proteins of *Bacillus thuringiensis*. *Microbiology Reviews* 53, 242–255.
- Jaoua, S., Zouari, N., Tounsi, S. and Ellouz, R. (1996) Study of the delta-endotoxins produced by three recently isolated strains of *Bacillus thuringiensis*. *FEMS Microbiology Letters* 145, 349–354.
- Kostichka, K., Warren, G.W., Mullins, M., Mullins, A.D., Craig, J.A., Koziel, M.G. and Estruch, J.J. (1996) Cloning of *cryV*-type insecticidal protein gene from *Bacillus thuringiensis*: the *cryV*-encoded protein is expressed early in stationary phase. *Journal of Bacteriology* 178, 2141–2144.
- Lereclus, D., Arantès, O., Chauvaux, J. and Lecadet, M.M. (1989) Transformation and expression of a cloned  $\delta$ -endotoxin gene in *Bacillus thuringiensis*. *FEMS Microbiology Letters* 60, 211–218.
- Masson, L., Erlandson, M., Puzstai-Carey, M., Brousseau, R., Juarez-Pérez, V. and Frutos, R. (1998) A holistic approach for determining the entomopathogenic potential of *Bacillus thuringiensis* strains. *Applied and Environmental Microbiology* 64, 4782–4788.
- Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning. A Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Sedlak, M., Waite, T. and Aronson, A. (2000) Regulation by overlapping promoters of the rate of synthesis and deposition into crystalline inclusions of *Bacillus thuringiensis* delta-endotoxins. *Journal of Bacteriology* 182, 734–741.
- Shin, B.S., Park, S.H., Choi, S.K., Lee, S.T. and Kim, J.I. (1995) Distribution of *cryV*-type insecticidal protein genes in *Bacillus thuringiensis* and cloning of *cryV*-type genes from *Bacillus thuringiensis* subsp. *kurstaki* and *Bacillus thuringiensis* subsp. *entomocidus*. *Applied and Environmental Microbiology* 61, 2402–2407.
- Tabashnik, B.E., Cushing, N.L., Finson, N. and Johnson, M.W. (1990) Field development of resistance to *Bacillus thuringiensis* in *Diamondback moth* (Lepidoptera: Plutellidae). *Journal of Economic Entomology* 83, 1671–1676.
- Taylor, R., Tippet, J., Gibb, G., Pells, D., Jordan, L. and Ely, S. (1992) Identification and characterization of a novel *Bacillus thuringiensis* delta-endotoxin entomocidal to coleopteran and lepidopteran larvae. *Molecular Microbiology* 6, 1211–1217.
- Tounsi, S., J'Mal, A., Zouari, N. and Jaoua, S. (1999) Cloning and nucleotide sequence of a novel *cryIAa*-type gene from *Bacillus thuringiensis* subsp. *kurstaki*. *Biotechnology Letters* 21, 771–775.
- Tounsi, S. and Jaoua, S. (2002) Identification of a promoter for the crystal protein-encoding gene *cryIIa* from *Bacillus thuringiensis* subsp. *kurstaki*. *FEMS Microbiology Letters* 208, 215–218.
- Zouari, N., Dhoubi, A., Ellouz, R. and Jaoua, S. (1998) Nutritional requirements of *Bacillus thuringiensis* subsp. *kurstaki* strain and use of gruel hydrolysate for the formulation of a new medium for delta-endotoxin production. *Applied Biochemistry and Biotechnology* 69, 41–52.
- Zouari, N., Ben Sik Ali, S. and Jaoua, S. (2002a) Production of delta-endotoxins by several *Bacillus thuringiensis* strains exhibiting various entomocidal activities towards lepidoptera and diptera in gruel and fish-meal media. *Enzyme and Microbial Technology* 31, 411–418.
- Zouari, N., Achour, O. and Jaoua, S. (2002b) Production of delta-endotoxin by *Bacillus thuringiensis* subsp. *kurstaki* and overcome of catabolite repression, by using highly concentrated gruel and fish-meal media in 2 and 20 dm<sup>3</sup> fermenters. *Journal of Chemical Technology and Biotechnology* 77, 871–876.